# Molecular ecology of the Kentish plover Charadrius alexandrinus

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# Molecular Ecology of the Kentish Plover *Charadrius alexandrinus*

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## Contents

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Each of you would deserve his own chapter in this thesis; I don't know where I would be without you.

Going to tropical beaches and study beautiful birds – what can I ask more for?!

I thank God every day for my privileged life.

## Summary

Molecular ecology has already provided profound insights into behaviour, ecology and systematics of organisms improving our understanding of the relationship between genetic variation and biodiversity. The objectives of my PhD were to develop new genetic markers and use these markers to address fundamental issues in evolutionary biology using shorebirds as model organisms. Shorebirds are part of the ancient avian *Charadriiformes* order and are characterised by extraordinary ecological and behavioural diversity. However, due to the lack of appropriate genetic markers the molecular ecology of many shorebirds has not been investigated previously. Therefore, first, I developed polymorphic microsatellite markers from genomic libraries for a behaviourally diverse shorebird, the Kentish plover *Charadrius alexandrinus* (*Chapter II*). Second, using the genomic data-bases I expended this work to develop further markers that cannot only be used in the Kentish plover, but also a large number of other shorebird species (*Chapter III*). Third, I investigated population differentiation and genetic diversity of Eurasian and American Kentish plover populations using the newly developed microsatellite markers and further mitochondrial markers (*Chapter IV*). The genetic differences between Eurasian and American populations that are mirrored by phenotypic differences call for a reconsideration of the current taxonomic status of the species; Eurasian and American populations should be recognised as belonging to two separate species. Finally, I asked how genetic diversity influences the fitness of precocial Kentish plover young (*Chapter V*). I found that survival of chicks until fledging was associated with genetic diversity (measured as heterozygosity) at three of eleven marker loci. Genetic diversity at one marker locus had a positive effect on survival whilst it had negative effects at two loci. The results of my PhD have brought up many new questions and I propose promising lines that need to be explored in the future (*Chapter VI*).

Chapter I

# Introduction

# Background

Molecular ecology, the application of molecular genetic methods to problems in ecology, is a broad but still young scientific discipline (Beebee & Rowe 2004). Most research in molecular ecology is centred around the molecule of life, deoxyribonucleic acid (DNA), attempting to link its variation with variation in ecology, behaviour and evolutionary history of individuals, populations and species. Once DNA sequences are known, ecologists and evolutionary biologists can answer many questions for which morphological, behavioural or ecological data cannot provide complete answers. Molecular ecology is bringing new insights into systematics, improving our understanding of the tree of life and how the present species phylogenies have evolved. It provides the tools for examining the coherence of populations, gene-flow between fragmented demes, and enables conservation biologists to identify genetic threats and model the survival prospects of populations. Molecular genetic tools have helped researchers to quantify reproductive success of individuals, reconstruct pedigrees and identify beneficial alleles and/or allele combinations to understand the interplay of natural and sexual selection. Its findings enhance our understanding of biodiversity and can help to conserve and protect it (Karp *et al.* 1997, Frankham *et al.* 2002, DeSalle & Amato 2004).

In molecular ecology various genetic markers are employed (Beebee & Rowe 2004). Popular markers currently used include *allozymes*, protein variants of a gene that can be separated by gel electrophoresis, *mitochondrial DNA markers*, haploid markers with intermediate variability that are inherited exclusively through maternal lineages, *amplified fragment length polymorphism (AFLP) markers,* restriction-digested DNA annealed to specific linkers which results in specific amplification band patterns, *single-nucleotide polymorphism markers*, homologous DNA fragments that differ in their sequence in a single base, *minisatellites,* 10-60 bp repeated long sequence motifs and *microsatellites,* 1-6 bp frequently repeated highly variable short sequences. Each marker has its strengths and limitations. To answer different questions the variability of different markers is exploited. Many markers were initially considered to be selectively neutral, although this assumption turned out to be often incorrect. For example, many microsatellites which are usually considered to be selectively neutral have been found to be located in expressed genes or in DNA regions with regulatory functions where their variability can modify functionality (Li *et al.* 2002, 2004).

**Microsatellites**

In my PhD I focussed on the application of microsatellite markers in shorebirds (plovers, sandpipers and allies, see below). Microsatellites (or simple sequence repeats) are the most versatile markers currently used in molecular ecology and can be applied to identify individuals, populations and species. Microsatellites are often found in non-coding regions of eukaryotic genomes (Ellegren 2000, Li *et al.* 2002). Variability is introduced by mutations and microsatellites exhibit high mutation rates of $10^{-3}$ – $10^{-7}$ allele changes per generation. However, these mutation rates differ between species and loci (Ellegren 2000, Buschiazzo & Gemmell 2006). The unique mutation process of a microsatellite involves changes of the repeat number by replication slippage or interchromosomal exchange (Buschiazzo & Gemmell 2006). Replication slippage, considered by most authors as the main mutation process, occurs when DNA strands are not properly aligned during the replication process. A new repeat is either added (when a loop forms in the newly synthesised strand), or deleted (when loops form in the existing template strands, Ellegren 2000, Buschiazzo & Gemmell 2006). Four repeat units are considered as the minimum for replication slippage to take place (Buschiazzo & Gemmell 2006), and the variability of microsatellites is associated with the length of the marker. Replication slippage is best characterised by stepwise mutation models (Ellegren 2000, Buschiazzo & Gemmell 2006). The resemblance of alleles at a given microsatellite locus is sometimes assumed to reflect their evolutionary history. Alleles of similar length are then closer related to each other than alleles with very different allele lengths (Coulson *et al.* 1998). The second mutation process, interchromosomal exchange, occurs during unequal cross-overs of homologous DNA strands, although the importance of this type of mutations is debated (Buschiazzo & Gemmell 2006).

Microsatellite markers are single locus markers that refer to the same genomic loci across all examined individuals. Each locus can be amplified by polymerase chain reaction (PCR) using primers that bind to up- and downstream target sequences surrounding the microsatellite. The products are visualised on gels or, when fluorescent primers are used for amplification, on automated genotypers. Microsatellites are codominant markers, and heterozygotes can be distinguished from homozygotes (assuming that all alleles can be amplified by PCR), because alleles differ in their length. However, due to the complexity of the mutation process in which both allele expansions and contractions occur, not all alleles of the same length are identical by

descent which can be a problem for applications such as estimating pairwise relatedness (Queller & Goodnight 1989, Lynch & Ritland 1999).

Although microsatellites are popular markers to investigate natural populations, the development of appropriate marker sets needs considerable funds and expertise. Their frequent occurrence in non-coding genomic sequences means they are little conserved and many primers work only in closely related species. Therefore microsatellite markers need often to be isolated, *de novo*, by screening enriched or unenriched libraries (Zane *et al.* 2002). Another popular way to find microsatellite markers is by testing available primers from related species for amplification and polymorphism in the species of interest ('cross-species amplification' tests, e.g. Primmer *et al.* 1996, 2005, Dawson *et al.* 2005). Success rates differ from species to species with some loci working across a broader taxonomy than others. However, the reasons for these differences are unknown.

Understanding the interaction between genetic diversity and fitness is a key element of evolutionary biology. Inbreeding with its negative consequences on fitness is detrimental for individuals and a main concern for conservation of populations and species (Charlesworth & Charlesworth 1987, Hedrick & Kalinowski 2000, Keller & Waller 2002). Microsatellite markers have been heralded as a pragmatic solution to measure inbreeding and relatedness in the wild because marker based estimates of relatedness should give close approximations to pedigree relationships without the need for long-term studies (but see Pemberton 2004, Csilléry *et al.* 2006). Marker based correlations between heterozygosity and fitness are frequently reported from wild populations (Coulson *et al.* 1998, Amos *et al.* 2001, Acevedo-Whitehouse *et al.* 2006, Ortego *et al.* 2007). However, the causes of the heterozygosity-fitness correlations are debated (Hansson & Westerberg 2002). In particular, it needs to be established whether observed correlations between marker heterozygosity and fitness are based on genomewide, direct or local effects and whether such correlations occur in both inbred and outbred populations.

**Why study shorebirds?**

Shorebirds are part of the *Charadriiformes,* an old avian order with approximately 350 species, that evolved about 80-120 million years ago (Baker *et al.* 2007). Recently the major relationships between the different *Charadriiformes* suborders have been thoroughly investigated with most of them being resolved (Thomas *et al.* 2004, Baker *et*

*al.* 2007). However, there are still insufficient data to resolve relationships between species (and populations) at the tips of the phylogeny (Thomas *et al.* 2004).

Shorebirds feature many diverse ecological characteristics. They breed on all continents including Antarctica; some species such as the black-winged stilt *Himantopus himantopus* and Kentish plover *Charadrius alexandrinus* have a cosmopolitan distribution that includes several continents, whereas others such as Chatham island snipe *Coenocorypha pusilla* and the St Helena plover *Charadrius sanctaehelenae* are restricted to a small geographic region that comprises a single island (Johnsgard 1981, del Hoyo *et al.* 1996).

Many shorebirds are migratory and their movements include record breaking long distance journeys. Arctic breeders may travel to their wintering habitats at the most southern tips of mainland America, Africa or Australia covering a distance of more than 20,000 km twice every year (van de Kam *et al.* 2004). A satellite tracked bar-tailed godwit *Limosa lapponica* was followed on an 11,570 km non-stop flight from Alaska to New Zealand in 8½ days, an achievement unmatched by any other animal (US Fish & Wildlife Service 2007).

Shorebirds also harbour an extraordinary diversity in breeding systems (Thomas *et al.* 2007). Some species are characterised by classic monogamy with both males and females providing care for the offspring. Others such as the ruff *Philomachus pugnax* show polygyny, where males meet for display competitions in leks and try to fertilise as many females as possible (e.g. the ruff, Pitelka *et al.* 1974, Oring 1982, del Hoyo *et al.* 1996). Finally, some shorebirds are polyandrous, where females are the dominant sex and mate with more than one male per season, or even reverse the classic sex-roles by becoming territorial and competing with other females for access to males (e.g. the bronze-winged jacana *Metopidius indicus* (Butchart 2000).

Although the ecological and behavioural diversity of shorebirds was already recognised by Charles Darwin (1871), when he used variation in shorebird breeding systems to illustrate sexual selection, the reasons for this diversity are still poorly understood (see also Székely *et al.* 2006). After several decades of research efforts in ecology it has become clear that this diversity cannot be explained by environmental differences between populations alone. For example, in some species several types of breeding systems occur in the same population: polygyny, polyandry and monogamy, e.g. Temminck's stint *Calidris temminckii* and Kentish plover (Hildén 1975, Lessells 1984,

Warriner *et al.* 1986). Instead genetic differences between individuals and populations may hold the key to explain this diversity and this is where molecular ecological investigations could bring new insights.

Photos by Clemens Küpper

**Figure 1.** Adult and freshly hatched Kentish plovers of the subspecies *nivosus* in Ceuta, Mexico. During the breeding season adult plumages are sexually dimorphic: the males exhibiting dark breast, head and eye stripes (left) whereas the ornaments of females are less pronounced (middle). The precocial chicks become highly mobile within hours after hatching when they leave the nest scrape, and are led by one or both parents to the feeding grounds.

The results of molecular ecological analyses will also have implications for shorebird conservation. Many shorebird populations are threatened and about four times more populations are declining than increasing (Stroud *et al.* 2004). Although the loss of genetic diversity is a major concern for the future of many species (Frankham *et al.* 2002), the genetic diversity of only a handful of shorebird species has been investigated up to date (Baker 2006). Moreover, most studies were conducted in arctic shorebirds and Baker (2006) argued that the observed low genetic diversity of these populations is due to severe bottleneck during the glacial periods. Detailed investigations into genetic diversity of populations from temperate or tropical zones have been hampered by the lack of appropriate markers such as microsatellites.

For my PhD I studied the molecular ecology of the Kentish plover. This small cosmopolitan shorebird belongs to the *Charadri* suborder, exhibits a large diversity in morphology and behaviour between and within populations (Box 1, Figure 1). Previous studies that used anonymous minisatellite probes as genetic markers revealed that genetic diversity could influence mate choice and suggested that the genetic mating

system is as diverse as the social mating system (Blomqvist *et al.* 2002, Küpper *et al.* 2004). However, due to the limitations of the available minisatellite markers the importance of genetic diversity on individual fitness and genetic differences between populations could not be evaluated by these studies and I addressed these important questions during my PhD.

**Box 1. Natural history of Kentish plovers *Charadrius alexandrinus***

The Kentish plover can be found in temperate to tropical zones of all continents (Johnsgard 1981, Hayman *et al.* 1986, del Hoyo *et al.* 1996). It forms an allopatric superspecies with white-fronted plover *C. marginatus* and red-capped plover *C. ruficapillus*. Six subspecies are usually recognised, the three American subspecies *C. a. nivosus*, *C. a. tenuirostris* and *C. a. occidentalis* are often called 'snowy plover' and their plumage is paler than that of the Eurasian subspecies (Hayman *et al.* 1986). The three remaining subspecies are found in Eurasia; the most abundant subspecies *C. a. alexandrinus* stretching from the Atlantic Coast in Western Europe to the Pacific coast in Eastern China and Taiwan. The other two Eurasian subspecies *C. a. seebohmi* and *C. a. dealbatus* are found in India/Sri Lanka and Japan/North East Asia, respectively.

Kentish plovers have a remarkably flexible breeding system that can include social and genetic polygyny, polyandry and monogamy within a single population (Lessells 1984, Warriner *et al.* 1986, Amat *et al.* 1999, Blomqvist *et al.* 2002, Székely *et al.* 2006). Both sexes have different plumages in the breeding season (Figure 1). As with other shorebirds, Kentish plovers produce small clutches with up to three eggs (Cramp & Simmons 1983). Both parents incubate the eggs, but soon after hatching one parent (usually the female) may desert the brood and attempts to remate. Several environmental factors that influence the breeding system have been identified. Biparental care is favoured when (i) competition for food is high (Kosztolányi *et al.* 2006) or (ii) both parents are needed to defend the young from predators (Amat *et al.* 1999). Conversely, uniparental care is favoured when environmental conditions are good, and one parent is enough to care for the young. The deserting parent often remates and can produce further offspring (Székely *et al.* 1999). Offspring survival can be easily quantified because Kentish plovers breed in open sparsely vegetated habitats. Offspring survival varies between populations and families. Male-biased broods survived better than female-biased ones in a Turkish plover population (Székely *et al.* 2004). The female-biased juvenile mortality may lead to male biased adult sex ratios favouring female desertion and polyandry (Székely *et al.* 2004). Offspring survival usually declines over the breeding season when environmental conditions deteriorate (Székely & Cuthill 1999).

Kentish plovers are protected by the Bern Convention. Populations have become increasingly fragmented with many of them declining. In North America the Pacific populations have been classified by the Federal Act of the US Fish and Wildlife Service as threatened and endangered. This is because of a sharp decline in the pre-1990s that was probably caused by increased human pressures on their breeding sites (US Fish & Wildlife Service 2001, Ruhlen *et al.* 2003).

## Objectives

My PhD had four main objectives:

1. *To develop new markers for the Kentish plover than can be used for estimating genetic diversity and relatedness of individuals and populations.* At the start of my PhD only 20 microsatellite markers were available for shorebirds (van Treuren *et al.* 1999, Thuman *et al.* 2002, Buehler & Baker, unpublished results), and 32 markers for the entire *Charadriiformes* order. For Chapter II I developed microsatellite markers for the Kentish plover from unenriched and enriched microsatellite libraries at the Sheffield Molecular Genetic Facilities.

2. *To develop universal genetic markers that can be used to address problems in molecular ecology across many shorebird species.* The availability of the first draft of an avian genome, the chicken *Gallus gallus* (International Chicken Genome Sequencing Consortium 2004) provided new possibilities to derive conserved genetic markers using genomic databases. In Chapter III I used the chicken sequence database and published sequences of *Charadriiformes* microsatellites in order to obtain microsatellite markers that are applicable to many *Charadriiformes* species.

3. *To investigate gene-flow, phylogeographic relationships and genetic diversities of Kentish plover populations.* In Chapter IV I examine morphological and genetic population differentiation of different plover populations in America, Africa and Eurasia using my previously developed microsatellite markers and further mitochondrial markers. I evaluate the current taxonomy of the Kentish plover subspecies and investigate genetic variation within different plover populations.

4. *To identify genetic bases of variation in offspring survival in shorebird populations.* Many environmental factors which influence offspring survival have already been identified in the Kentish plover (Box 1). However, whether there is also a genetic prevalence affecting offspring survival has not been investigated in shorebirds before. In Chapter V, I examine the associations between microsatellite heterozygosity and chick survival and discuss whether genomewide, local or direct effects describe best the observed heterozygosity-fitness associations.

Finally, *en route* to tackle these fundamental issues in molecular ecology, I devoted some of my efforts to the study of breeding systems and the cooperation of Kentish plover parents to bring up their offspring (see Appendices I - IV). This work is timely,

because recent theoretical and empirical results are opening new research lines to investigate sexual selection and the evolution of cooperation and breeding systems (e. g. Arnqvist & Rowe 2005, Houston *et al.* 2005, Thomas *et al.* 2007).

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Chapter II

# Characterisation of 36 polymorphic microsatellite loci in the Kentish plover (*Charadrius alexandrinus*) including two sex-linked loci and their amplification in four other *Charadrius* species

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**Contributions:**

**C. Küpper:** primer design & testing, analyses of genotypes, manuscript preparation
**G.J. Horsburgh:** set up and screening of genomic libraries, primer testing
**D.A. Dawson:** supervision of genetic work, primer design, analyses of genotypes, manuscript improvement
**R. ffrench-Constant:** advice on DNA extractions & advice on initial cross amplification testing
**T. Székely:** sample acquisition, manuscript improvement
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Chapter III

# Enhanced cross-species utility of conserved microsatellite markers in shorebirds

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**D.A. Dawson:** study design, manuscript improvement

## Abstract

**Background:** Microsatellite markers are popular genetic markers frequently used in forensic biology. Despite their popularity, the characterisation of polymorphic microsatellite loci and development of suitable markers takes considerable efforts. Newly available genomic databases make it feasible to identify conserved genetic markers. We examined the utility and characteristics of conserved microsatellite markers in *Charadriiformes* (plovers, sandpipers, gulls and auks). This order harbours many species with diverse breeding systems, life histories and extraordinary migration biology whose genetics warrant investigation. However research has been largely restrained by the limited availability of genetic markers. To examine the utility of conserved microsatellite loci as genetic markers we collated a database of *Charadriiformes* microsatellites, searched for homologs in the chicken genome and tested conserved markers for amplification and polymorphism in a range of *Charadriiformes* species.

**Results:** Sixty-eight (42 %) of 161 *Charadriiformes* microsatellite loci were assigned to a single location on the chicken genome based on their E-value. Fifty-five primers designed from conserved microsatellite loci with an E-value of E–10 or lower amplified across a wider range of *Charadriiformes* species than a control group of primers from ten anonymous microsatellite loci. Twenty-three of 24 examined conserved markers were polymorphic, each in on average 3 of 12 species tested.

**Conclusion:** Genomic sequence databases are useful tools to identify conserved genetic markers including those located in non-coding regions. By maximising primer sequence similarity between source species and database species markers can be further improved and provide additional markers to study the molecular ecology of populations from non-model organisms.

## Background

Microsatellites or short sequence repeats (SSRs) are relatively small 1-6 basepair (bp) tandem repeats that are found in the genomic DNA of pro- and eukaryotes. Although the majority of microsatellites are located in non-coding sequences [1,2] and considered to be selectively neutral, some microsatellite loci are located in functional regions and involved in chromatin organisation, regulation of gene activity and metabolic processes such as DNA replication and recombination [3]. Microsatellites exhibit high mutation rates of $10^{-2}$ to $10^{-6}$ per locus per generation [4]. The main mutational processes responsible for the variability in microsatellites are considered to be replication-slippage

and recombination [3]. Both processes change the length of the microsatellite by altering the number of repeats of the microsatellite. A number of microsatellite characteristics have been associated with differing microsatellite variability. Mutation rates of microsatellites have been found to be taxon-specific [4-6]. Microsatellite variability covaries with allozyme diversity of a taxon [7]. Repeat length can predict the variability and stability of a microsatellite with longer loci found to be more variable, but also more unstable than shorter ones [4,5,7-9]. The type of microsatellite motif may affect abundance and variability. For example it was found that microsatellites with tri- or hexanucleotide motifs are more frequent in coding than in non-coding regions, possibly because mutations of microsatellite in coding regions will not lead to deleterious frameshift mutations [1,2].

The high variability and simple processing through polymerase chain reactions (PCRs) lead to the frequent application of microsatellite markers in studies on natural populations. However, one obstacle for the wider application of microsatellites is the difficulty in the development of a sufficient number of suitable markers for a given species. Although microsatellites are ubiquitous in eukaryotic organisms, their abundance varies across different groups [2]. Microsatellites are less common in birds compared to other vertebrates [7,10]. There are two principal strategies to obtain microsatellite markers. First, microsatellite markers can be developed by screening enriched or unenriched microsatellite libraries [11]. Success rates differ according to protocol and taxa, although usually a medium to high number of polymorphic markers can be isolated using this approach. Since microsatellites are at such a low frequency in birds their isolation is most efficient using enrichment protocols. This involves many stages, is a skilled and time consuming process and requires considerable economic funds and a well-equipped molecular laboratory which are not always available in ecological and conservation research.

The second method makes use of existing microsatellite markers isolated in different species to the species of interest (target species). For cross-species amplification tests ("transferability" [9,12]) existing primers developed in related species are tested for amplification and polymorphism in the target species. One drawback of cross-species amplification is that success rates decline with evolutionary distance between the target species and source species [9,13-15]. In birds, the most microsatellite markers have been developed for orders *Passeriformes* and *Galliformes*. *Passeriformes* is a species-

rich relatively recent clade [16] for which more than 550 microsatellite markers exist [17]. Several studies have successfully identified additional polymorphic loci by cross-species testing in birds. Development of whole primer sets from cross-species amplification tests has been successful in *Falconiformes* [18], *Galliformes* [19-21] and *Passeriformes* [15,22-24]. However, in many other avian orders fewer microsatellite loci have been isolated and therefore the development of microsatellite markers by testing loci from other species is limited.

Cross-species amplification success varies not only between taxonomic groups, but also among microsatellite loci. Although many markers fail to amplify even in closely related species, some markers have higher utility than others [9,13-15,25,26], DA Dawson, unpubl and BIRDMARKER webpage (http://www.shef.ac.uk/misc/groups/molecol/deborah-dawson-birdmarkers.html). A few loci, such as *HrU2* [26], *LEI160* [27], *LOX1* [28] and *Man13* [29] can be almost universally amplified across the avian taxonomy (*HrU2* & *LOX1*: [9,13], *LEI160,* DA Dawson unpubl; *Man13*: DA Dawson and G Hinten, unpubl, see also BIRDMARKER webpage). This suggests that some loci are more conserved than others. Since the degree of microsatellite conservation is usually not known at the time of their isolation, identifying conserved primers usually involves extensive primer testing and only few conserved markers have been identified to date.

The *Charadriiformes* order (sandpipers, plovers, gulls and auks) is an ancient monophyletic avian order of 365 species [30] that probably evolved around 79–102 million years ago [31]. Recently the *Charadriiformes* have become the focus of a number of studies in evolutionary biology because they harbour many species with an unusual diversity in mating and parental care strategies, flight metabolism, migratory behaviour and sexual size dimorphism [32-36]. Appropriate genetic markers would help to increase the understanding of e.g. the evolution of breeding systems and the connectivity between populations, but markers are available for less than 15 *Charadriiformes* species. Additionally, many shorebird populations are declining and genetic markers are needed to monitor and manage conservation effectively.

In this study, we examine the potential of utilising the available published *Charadriiformes* microsatellite sequences and the sequenced chicken genome to identify conserved *Charadriiformes*-chicken microsatellite loci. Initially, we mapped

conserved *Charadriiformes* microsatellites on the chicken genome. Secondly, we explored their cross-species utility across members of the order *Charadriiformes*. One concern is that conserved microsatellite loci are located in functional genomic regions and exhibit low or no polymorphism. Therefore we compared cross-species polymorphism and heterozygosity levels across different *Charadriiformes* species. Third, we examined correlates of cross-species amplification success and polymorphism to predict the utility of other conserved microsatellite loci.

## Results

### Mapping

Sixty-eight *Charadriiformes* microsatellite sequences were assigned to a location on the chicken genome based on sequence homology (with E-values ranging between E–6 to E–121). Two further sequences (*BmaCCAT443* and *BmaGATA464*) hit to an unknown chicken homolog which had not yet been assigned to a chromosomal region. Sixty-four sequences were assigned to fourteen autosomal chicken chromosomes and four to the Z chromosome (*Calex-26*, *BmaTATC353, Apy09* and *Mopl3*; Table 1, Figure 1). The mapping of loci assigned to the Z chromosome was validated by analysing the genotypes of birds with known sex (including males and females). A location on Z was supported if all females were homozygous whilst at least some males exhibited heterozygous genotypes. This was found in all markers assigned to the chicken Z chromosome: *Calex-26* (based on 42 Kentish plovers [37]), *BmaTATC353* (based on genotyping of 15 marbled murrelets, Z Peery, pers. communication), *Mopl3* (126 genotyped mountain plovers, SJ Oyler-McCance and J St. John, pers. communication) and *Apy09* in whiskered auklet (24 genotyped whiskered auklet individuals, DA Dawson and FM Hunter, unpubl).

The microsatellite motif of the *Charadriiformes* sequences was not always retained in chicken when we examined the microsatellite loci that were mapped to the chicken genome ($N = 68$). A comparison with the chicken genomic sequences revealed that the same microsatellite repeat motif was present in 32 (47 %), a different microsatellite motif was found in 10 sequences (15 %) whilst no microsatellite repeat motif was found in 26 (38 %) of the sequences (Table 1).

## Cross-species amplification

In total we tested 55 'standard primers' (see Methods) from different conserved microsatellite sequences and 10 primers from anonymous microsatellite sequences. In both groups a similar proportion of microsatellite loci was isolated in each of the three test species representing the orders *Charadri, Lari* and *Scolopaci* (chicken-*Charadriiformes* conserved loci: 13 of 55, anonymous loci: 3 of 10, $\chi^2$ test: $\chi^2 = 0.0161$, $df = 1$, $P = 0.899$). In 17 of 55 conserved sequences we obtained a specific product for all three species, whilst we didn't obtain specific products for any of the anonymous sequences in all test species. When we compared the proportion of species in which a primer amplified, primers designed from conserved sequences outperformed primers designed from anonymous sequences significantly (Figure 2a, amplification success: $\text{median}_{\text{conserved}} = 0.667$, $\text{median}_{\text{anonymous}} = 0.167$, Wilcoxon rank sum test: $N = 65$ (55/10), $df = 1$, $W = 469$, $P < 0.001$).

For 24 conserved sequences we designed a second consensus primer set with primer binding sites in the conserved regions of the flanks. Cross-species amplification rates were higher for consensus primers than standard primers for the same microsatellite sequence (Figure 2b, Wilcoxon matched pair test: $N = 24$, $df = 1$, $V = 96$, $P = 0.006$). Amplification success increases when the annealing temperature is reduced [38]. Hence, the reason for the improvement of amplification could have been that consensus primer were designed and tested at lower annealing temperatures (consensus primers: 50–62 °C, standard primers: 54–66 °C). However 19 out of 24 (83 %) consensus primers amplified best at annealing temperatures of 54 °C. Also, the difference in amplification success between consensus and standard primers remained significant (Wilcoxon matched pair test: $N = 19$, $df = 1$, $V = 41.5$, $P = 0.023$) when only the 19 loci where both primers amplified in the maximum number of species at 54 °C and more were analysed.

Twenty-three of 24 consensus primer pairs exhibited between one and three base-pair mismatches between chicken and *Charadriiformes* primer sequences. Each mismatching primer base was replaced by a suitable degenerated base which included both of the possible bases. The use of degenerated bases will dilute the effective concentration of the primer with the highest target affinity which could lead to higher amplification failure. However amplification success was not related to the number of

degenerated bases per primer pair (Kruskal Wallis test: $N = 23$ (6/12/5), $df = 2$, $\chi^2 = 1.1978$, $P = 0.549$).

The final model to identify correlates of amplification success contained a single explanatory variable (Table 2). Cross-species amplification success was significantly associated with the E-value of a given sequence among conserved sequences (Figure 3). E-values for primers that amplified in all tested species ranged from E–110 (*Mopl18*, Table 1) to E–21 (*BmaTATC371*). Standard primers from sequences with lower E-values amplified in more *Charadriiformes* species than those with higher E-values (Generalised Linear Model (GLM) with binomial error structure: $df = 53$, $B = –0.02$, $t = –2.58$, $P = 0.013$).

## Allelic variability and observed heterozygosity

Twenty-three of 24 conserved microsatellite loci exhibited two or more alleles in on average 3 of the 13 species tested (Table 3, range 1 to 8 species per locus). There was considerable variation among species in regard to the number of polymorphic markers. Excluding the markers that had been isolated in the target species we found on average 7 of 24 markers per species (range 0–11 polymorphic loci/species) to be polymorphic when tested in four unrelated individuals from the same population (Figure 4). None of the 23 markers included in polymorphism tests were sex-linked based on the distribution of alleles in known male and female birds (individuals were sexed using P2/P8 primers [39]) and based on their predicted chromosome location based on the chicken genome map (Figure 1).

Allelic variability (the proportion of species in which a microsatellite was polymorphic) was significantly associated with three factors: i) microsatellite motif, ii) repeat length and iii) whether the microsatellite was interrupted or not (Table 2). Microsatellite loci with dinucleotide motifs were variable in more species than those consisting of tetranucleotides (GLM with quasibinomial errors: $df = 19$, $B = –1$, $t = –2.15$, $P = 0.045$). Microsatellites with longer repeat regions were polymorphic in more species than those with shorter repeat regions (GLM with quasibinomial errors: $df = 19$, $B = 0.02$, $t = 2.22$, $P = 0.039$). Interruption of the microsatellite repeat regions marginally reduced the proportion of species in which a locus was polymorphic (GLM with quasibinomial errors: $df = 19$, $B = -1.02$, $t = –2.09$, $P = 0.051$). Note that genetic distance between source and target species (based on $\Delta T_mH$ DNA-DNA hybridisation

value taken from [40]) was not included in this model since the response variable in this model was the proportion of species in which a locus was found to have more than one allele.

When we examined variability of primers in the larger sample of Kentish plover, whiskered auklet and ruff (Table 4) we found that average observed heterozygosities combined in all three test species was lower compared to observed heterozygosities of species in which a microsatellite had been originally isolated and characterised (Wilcoxon matched pair test: $N = 23$, $V = 226$, $P < 0.001$). Heterozygosity in all three test species declined with increasing genetic distance to the microsatellite source species, and the decline was highly significant in all three species combined (Figure 5, Generalised Linear Mixed Model [GLMM]: $df = 33$, $B = -0.04$, $t = -7.59$, $P < 0.001$). An alternative model excluding those loci that had been developed for the target species gave the same qualitative results (model not shown).

## Discussion

We have shown that sequence information from annotated genomes can be used to identify and map conserved microsatellite loci that are located in both translated and non-translated regions. Our study has two major findings. Firstly, primers designed from conserved microsatellite loci amplify across a wider taxonomic range than those derived from anonymous microsatellite loci. Second, when highly conserved regions of the flanks of a microsatellite are used as the primer binding sites amplification success can be further improved.

### Correlates of cross-species amplification

Amplification success was not associated with genetic distance between microsatellite source species and test species in this intraorder analysis of *Charadriiformes* microsatellite markers. The E-values obtained from blast searches served as a better predictor for the width of taxonomic range in which a microsatellite could be amplified.

Using 1147 primers derived from conserved regions of the human genome Housley et al. [41] identified the number of primer mismatches and primer GC content as factors that predicted amplification success in mammals. In contrast to our study, Housley et al. investigated amplification success using generally conserved sequences in mammals and did not specifically use variable loci such as microsatellite loci.

Secondly, they aligned genomic sequences from humans with genomic sequences from dog, rat or mouse to perform intragroup-comparisons of amplification success whilst we used an outgroup taxon (chicken) for sequence alignments and comparisons. Thirdly, our sample size was much smaller than the one used for the intra-mammalian comparison due to the small number of *Charadriiformes* microsatellite loci available. The results of both studies are very similar despite the large differences in study design. Sequence conservation (represented by the E-value) was the main predictor for amplification success of a genetic marker. Most loci with an E-value lower than E–20 amplified in all species tested suggesting that this could be a critical value indicating the utility of a marker for cross-species amplification within the order *Charadriiformes*. Consensus primer sets that had been designed to include the smallest number of mismatches between sequences amplified better than standard primers for which no action to counteract the presence of mismatches had been taken. Amplification success of consensus primers was generally very high across the three *Charadriiformes* suborders (Figure 2 B).

The number of mismatches per primer pair did not affect amplification success among the consensus primers for a number of possible reasons. First, we restricted the sequence mismatches between chicken and *Charadriiformes* for consensus primer pairs to a maximum number of three. Second, we introduced degenerated bases to account for those mismatches. According to our primer design rules we attempted to minimise the number of mismatches and positioned mismatches away of the 3' end for a given primer, although the position of the mismatch/degenerate base did not affect amplification success significantly (data not shown). Third, inclusion of degenerated bases might have led to amplification failure due to the lack of suitable primer. However we did not observe such failures, probably because the primer concentration in our tests was relatively high (1µM, see Methods). Thus the amplification results of our consensus primers should not be used to cast doubts on the importance of high primer sequence similarity for amplification success.

Primmer et al. [13] proposed that the proportion of microsatellite loci that can be amplified declines with increasing genetic distance between source and target species. Our results suggest that the slope of the amplification decline is predominantly locus specific and will largely depend on the conservation of the flanking sequences surrounding the microsatellite repeat. Hence microsatellite loci with highly conserved

flanks can be amplified in more distantly related species whilst those with non-conserved flanks may be useful only in a very narrow taxonomic group.

### Polymorphism

Polymorphism rates of conserved loci varied greatly between species. The proportion of species in which a microsatellite exhibited one or more alleles was associated with microsatellite repeat length, motif and whether it was interrupted or not. Our results are consistent with previous theoretical and empirical studies that examined the effect of these microsatellite properties on mutation rate [4-6,8] and polymorphism [7,9]. A positive association between repeat length and polymorphism was found empirically in other vertebrates, arthropods and plants [4,7-9]. Dinucleotide microsatellite loci exhibited higher mutation rates than tetranucleotide microsatellite in mice and yeast [5]. In humans and chimpanzees, microsatellite loci with interrupted repeat regions had a two-fold decrease in the mutation rate which was interpreted that interruptions could reduce the opportunities for replication slippage [6].

Although we showed that amplification success of existing microsatellite markers can be improved by redesigning primer sets, heterozygosity rates were lower in test than in source species. Polymorphism declines faster with evolutionary distance than amplification success [14]. A possible explanation is that polymorphism of many loci evolved in the recent evolutionary past and therefore is confined to a relatively narrow phylogenetic taxon. This argument is supported by findings of other studies in amphibians, birds and mammals [9,14] which show that polymorphism rates drop fast with increasing evolutionary distance between source and target species. However the steepness of the decline appears to be locus specific. There was large variation in the width of the taxonomic region for which a given microsatellite was polymorphic. For instance the locus *BmaTGAA523* was polymorphic in only two of twelve species when tested in four unrelated individuals, whilst another locus, *BmaTATC371* isolated in the same species and with a similar repeat length, was polymorphic in seven of twelve species tested. Of the 24 *Charadriiformes* microsatellite markers we tested, a median of 7 markers were polymorphic per species (12 *Charadriiformes* species tested). Five and more polymorphic markers were found in five of the six test species where previously no microsatellite markers had been identified. For another six species where markers had already been characterised we found between three and 11 new polymorphic markers. The number of species in which these 23 markers are useful is likely to

increase because we tested them only in 12 out of 365 *Charadriiformes* species. Finally, we assessed the variability of markers only within a single population to make the level of polymorphism comparable to the polymorphism in the source population. Some markers that we found to be monomorphic may exhibit population specific polymorphism or have different alleles fixed in different populations and turn out to be useful to investigate population differentiation.

The observed decline of polymorphism in relation to the genetic distance to the source species could be partly explained by the selection process during the isolation of microsatellite sequences ('ascertainment bias hypothesis', [7,42]). During the construction of microsatellite libraries typically long microsatellite sequences with 10–30 repeat units are selected to maximise the probability that a locus is polymorphic in the species in which it is developed. Moreover only the sequences of the polymorphic loci are normally submitted to sequence databases which means that monomorphic loci with fewer repeat units in the source species are lost because they are not reported. However repeat expansion and microsatellite polymorphism are likely to be a result of the recent phylogenetic history. Therefore submitting the sequences of monomorphic loci to genomic databases should lead to identification of more conserved markers and the development of useful markers through cross-species amplification.

Differences in the degree of microsatellite polymorphism among species are not exclusively attributable to recent divergence in microsatellite evolution. Genetic diversity, which is often reflected by microsatellite polymorphism varies among populations and species. Low microsatellite polymorphism can indicate depleted genetic variability due to bottlenecks, genetic drift or inbreeding. If genetic diversity for a given population is low, a combination of screening of hundreds of microsatellite loci and the development of microsatellite markers using the conventional library approach may be needed to find a suitable set of polymorphic markers.

Only a handful of shorebird populations have been investigated for genetic diversity. Low genetic variability of allozymes and mitochondrial control region has been found in several species of sandpipers that breed in the high arctic and it has been hypothesized that historical population fluctuations that occurred during and after glaciations are responsible for this low genetic diversity [43]. In our study the greater sheathbill showed the least genetic diversity being monomorphic at all 23 microsatellite

loci that we examined (Table 3). Greater sheathbills breed exclusively in the Antarctic, where they live as scavengers close to other bird colonies. Current population estimates give a stable total number of approximately 20,000 sheathbills [44], but past climate fluctuations may have lead to a small effective population size similar to those of arctic breeders. Thus the low observed microsatellite diversity might reflect a recent recovery. Alternatively, the evolutionary distance between sheathbills and the source species from which we derived the tested microsatellites is too large with all polymorphism being depleted. Different genetic markers such as markers from the mitochondrial control region, other microsatellite markers or highly variable nuclear genes such as genes of the major histocompatibility complex need to be examined to answer whether the low microsatellite variability truly reflects general low genetic diversity in sheathbills.

Contrary to sheathbills, whiskered auklets and Kentish plovers showed the highest genetic diversity in our analysis. In Kentish plover and whiskered auklet, twelve of the 23 microsatellite loci tested were found to be polymorphic when tested in four individuals. Excluding the markers which had been isolated in both species this lead to ten (Kentish plover) and eleven (whiskered auklet) newly described polymorphic markers. Both species live in very different habitats and geographic locations. Whiskered auklets are pelagic feeders that inhabit a number of small islands in the Northern pacific, whilst Kentish plovers are cosmopolitans and found at beaches and saline lakes in temperate and subtropic regions [45]. The high genetic diversity in both species is reflected in high levels of observed heterozygosities at microsatellite loci that had been isolated in these species by cross-species amplification (this study) and when loci were isolated from an enriched genomic library [14,37]. High variability of many microsatellite loci in these species suggests that depletion of genetic variation is not a general characteristic of *Charadriiformes* order, but rather an attribute of certain species or populations due to their historic demography and phylogeography.

The possibilities for the application of conserved markers go beyond examining genetic diversity. Polymorphic conserved markers can be used, for example, to investigate chromosomal organisation by constructing linkage maps [46,47]. A major advantage of conserved over conventional markers is that the same makers can be used to investigate and so compare chromosomal structure and genomic organisation between several different species [17,48].

Preservation of flanking regions is often caused through a direct functional role or linkage disequilibrium with functional genomic regions (e.g. fitness relevant genes, [49]). Selection pressures may affect variability of a locus by either restricting polymorphism [1,2] or promoting polymorphism if variability is adaptive [50]. This can be problematic for applications of genetic markers that rely on neutrality. However, for common applications such as parentage assignment or estimating relatedness such markers will be useful. Furthermore, if a marker is found to be associated with a locus that is under selection, its function can be explored and changes or retention of functionality can be compared under different environmental conditions, across different populations and/or taxa.

The conserved markers we designed and characterised can be most conveniently handled in the laboratory. All polymorphic primers amplify at similar PCR conditions ($T_a$ 54–55 °C, 2.0 µM $MgCl_2$ concentration) which may facilitate i) quick and economic screening for amplification and polymorphism in new target species and ii) efficient processing since several loci can be run together in a single multiplex PCR.

### Dealing with null alleles

Five of the 24 primer sets which were tested for heterozygosity had high null allele frequency estimates (≥ 0.1, CERVUS 2.0) in one of the three test species (Kentish plover, whiskered auklet and ruff). Subsequent analyses showed that these loci were not in Hardy-Weinberg equilibrium (Table 4). There was no obvious relationship between the failure to comply to Hardy-Weinberg equilibrium and the number or position of degenerated bases in each primer pair. Null alleles arise if the primer sequence does not match the target sequence of a given allele and the allele therefore fails to amplify. If not corrected for, presence of null alleles may interfere with algorithms to estimate relatedness [51]. Sequencing the locus in the study species and redesign of primers would prevent the occurrence of null alleles. The effect of null alleles may also be reduced computationally. If the proportion of null allele is low their impact on relatedness estimates can be reduced by using maximum likelihood correction methods when computing relatedness relationships [52].

### Development of conserved markers in other avian groups

*Charadriiformes*, chicken and most other modern birds belong to the avian group of the Neognathae. Recent molecular data suggest that the chicken group of *Galliformes* together with *Anseriformes* form a sister taxon to the other Neognath birds [53] and

therefore have the same phylogenetic distance to all Neognathae. Flanking regions of about one in seven *Charadriiformes* microsatellite loci were found to be conserved in chicken. Since the proportion of microsatellite homologs is likely to be associated with the phylogenetic distance between genomic resource species and source species, we expect a similar proportion of conserved microsatellite loci to be found between chicken and other Neognath groups to that which we found between chicken and *Charadriiformes*. In fact, for taxa that are closer related to chicken (e.g. *Anseriformes*) we predict an even higher success rate of identifying suitable microsatellite markers through data mining.

Genomic sequences of further organisms will facilitate the use of already characterised microsatellite loci for identification of microsatellites with suitable flanks for the development of consensus primer sets. In birds, the sequencing of the genome from another Neognath bird the zebra finch (*Taeniopygia guttata*) is close to completion (expected autumn 2008). Zebra finches are phylogenetically closer to *Charadriiformes* and other Neognath birds than are chicken, hence more microsatellite homologs and conserved markers might be obtained using zebra finch sequences as a reference.

## Conclusion

We have shown that sequence information available from genomic databases can be used to enhance the utility of microsatellite markers for studies of evolution and conservation, even for taxonomic groups where little sequence data is available. Sequence information of translated and untranslated parts of the genome are useful for comparing and designing consensus primers even when they involve genetically distant relatives such as *Charadriiformes* and *Galliformes* species. Cross-species amplification tests can be carried out more efficiently by identifying and utilising conserved microsatellite loci that will amplify across a broader taxonomic range. By selecting highly conserved regions of the microsatellite flanking sequence for primer design the number of species in which a locus will amplify can be increased even further. Markers derived from conserved loci with an E-value of E–20 or lower amplified across the entire order. Our findings will facilitate marker use for species where no markers have been identified yet and for species where more markers are needed. To date, 21 vertebrate and 20 invertebrate genomes have been sequenced and fully assembled (source: www.genome.ucsc.edu, May 2008). This number is expected to increase rapidly with decreasing sequencing costs and the application of new more efficient

sequencing techniques. The methodology we have outlined will make it possible to extend population genetic and evolutionary studies to other non-model species that have been previously neglected because the lack of sufficient genetic markers.

## Methods

### Blast search

We searched for available nuclear microsatellite sequences isolated in *Charadriiformes* species that were deposited before 15th of July 2006 in the nucleotide sequence databases of GenBank, DNA data bank of Japan, and European Molecular Biology Laboratory (EMBL) through the EMBL web portal (http://www.ebi.ac.uk/ebisearch/) using the key words "Charadriiformes microsatellite" and "Charadri* microsat*". Additionally for one species (oystercatcher *Haematopus ostralegus)* eight microsatellite primers of polymorphic loci had been published [54] but the microsatellite sequences were not found in the EMBL database. In this case the authors (R. van Treuren et al.) generously provided the sequences of the eight polymorphic and 29 further unpublished monomorphic oystercatcher microsatellite loci which were then submitted to EMBL in agreement with the authors (accession numbers: AM600643-AM600679).

Only microsatellite sequences that were polymorphic in the source species and had sufficient flanking sequence for primer design were considered (i. e. a minimum of 30 bp of flanking sequence on either side of the repeat motif). In total we found 163 suitable microsatellite sequences (Supplementary material). All sequences were checked for duplicates using the MegaBLAST program available from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/ [55]). Four pairs of homologs were found (K32/LarsZAP26, K56/LarsZAP19, LarsNX24/Rbg27 and LarsZAP11/Rbg29). LarsZAP26 and K32 were identical duplicates and the primer set was designed from K32. For the remaining duplicates the shorter sequence of each pair was dropped from the analysis (LarsZAP19, LarsNX24, LarsZAP11).

We identified homologous *Charadriiformes* microsatellite loci in the chicken *Gallus gallus* as follows. Unique *Charadriiformes* microsatellite sequences were compared against the chicken genome database v2.1 (WASHUC 1, Version e! 41, available at http://www.ensembl.org/Gallus_gallus/) using a WU-BLAST (Gish W. 1996 – 2004; http://blast.wustl.edu) implemented in the Ensembl browser with the "genomic

sequence (masked)" and "distant homologies" settings. The E-value was used as a measure of *Charadriiformes*-chicken homology. It is mainly characterised by sequence length of the query sequence and its similarity to the homolog in the database. In absence of duplications and gene orthologues lower E-values represent higher probability of sequence homology. All sequences from which both flanking regions had hit the chicken genome with E-values lower than E–10 were classified as conserved sequence homologs. Microsatellite sequences with only one flank hitting were not considered. In total we identified 55 *Charadriiformes* microsatellite sequences with homologs present in chicken genome with an E-value of ≤ E–10 and both flanks hitting to the same location (Supplementary material).

## Mapping of *Charadriiformes* microsatellite sequences on the chicken genome

We adapted the Blast methods from [48] to map the *Charadriiformes* sequences to the chicken genome:

i) all sequences hitting with either both flanks at an E-value ≤ E–5,

ii) all sequences that hit with one flank at an E-value ≤ E–10 (cf [48]: E–5),

iii) sequences that hit several times at different locations in the genome were mapped only if the E-value of the best blast hit (lowest E-value) was higher by ≥E–5 than the next blast hit (cf [48]: E–10).

In total 68 *Charadriiformes* microsatellite sequences were mapped on the chicken genome (Figure 1) using the programme MAPCHART [56]. The recorded locations of centromere are based on the regions of highest GC content on the chromosome (following [17]; data obtained from the NCBIs *Gallus gallus* Build 2.1: http://www.ncbi.nlm.nih.gov/genome/guide/chicken/index.html)

## Cross-species amplification rates of conserved and anonymous microsatellites

To examine whether cross-species amplification was affected by presence or absence of a chicken homolog for a given sequence we designed new primer sets ('standard primers') for a total of 65 loci using PRIMER3 [57]. We did not use already published primers developed in different laboratories because primer design methods can be very heterogeneous between different laboratories and this may have compromised our results [9]. We randomly selected ten microsatellite loci that had hits with an E-value of E–10 or higher of only one flank (anonymous sequences, Table 1) and compared

amplification success with the success of conserved chicken-*Charadriiformes* loci in which both flanks hit at the same location in the chicken genome with an E-value of E-05 or lower. For the design of standard primers, we used default options of PRIMER3 with the following adaptations:

i) the melting temperature ($T_m$) between 50 and 65 °C, with 62 °C as the preferred $T_m$,
ii) $T_m$ difference between forward and reverse primer < 0.5 °C,
iii) we manually checked for even distribution of all four nucleotide bases,
iv) a primer GC content of 20 – 60 %,
v) a product size between 70 and 450 bp.

The reverse primers of seven of the eleven Kentish plover *Charadrius alexandrinus* and two of the five whiskered auklet *Aethia pygmaea* loci were ordered with "GTTTCTT"-'pigtails' to reduce stutter bands [58]. The forward primer of each pair was labelled with a fluorescent label, either FAM or HEX.

Following [40,59] we recognise three major *Charadriiformes* lineages: *Lari*, *Scolopaci* and *Charadri*. All primers were tested for amplification success in one candidate species from each *Charadriiformes* lineage: whiskered auklet (for suborder *Lari*), ruff *Philomachus pugnax* (suborder *Scolopaci*) and Kentish plover (suborder *Charadri*). The suborders are separated by a genetic distance of 15.6 *Charadri/Lari* - *Scolopaci* and 12.8 *Charadri* - *Scolopaci* ($\Delta T_m H$ DNA-DNA hybridisation value [40]). All primer sequences are provided as supplementary table (Supplementary material).

DNA was extracted from blood samples that were stored either in Queen's lysis buffer [60] or absolute ethanol. One of three extraction methods was used; an ammonium acetate method [61], a sodium acetate method [62] or an adapted phenol-chloroform method [63]. All samples were visualised on a 0.8 % agarose gel stained with SYBRsafe (Invitrogen) to check for DNA quality. DNA concentration was estimated by measuring the optical density of a sample at 260 nm using a fluorimeter. Each sample was checked for amplification prior to tests using the *LEI160* primer set [27], a marker that amplifies across all of approximately 100 various bird species tested to date (DA Dawson, unpubl).

Each 10-µl PCR contained approximately 10 ng of DNA and 0.25 units of Taq DNA polymerase (Bioline) in the manufacturer's buffer with a concentration of 1.0 µM of each primer, 2.0 µM $MgCl_2$ and 0.20 mM of each dNTP. Loci were amplified by PCR using a thermal cycler (MJ Research model PTC DNA engine) and the following program: one cycle of 3 min at 94 °C followed by 35 cycles at 94 °C for 30 s, annealing temperature (temperature gradient from 54 – 66 °C) for 30 s, 72 °C for 30 s and a final extension cycle of 10 min at 72 °C. PCR products were visualized on 2 % agarose gel stained with SYBRsafe (Invitrogen) to check for amplification success. Amplification success was a binary variable, which we defined as 'successful' if a single clean band could be visualised on the gel, multiple band patterns and no products were recorded as 'failed'.

### Cross-species amplification rates of consensus and standard primers

In addition to the standard primers, we designed a second pair of consensus primers with a minimum number of mismatches between chicken and shorebird sequences. Within microsatellite flanks the degree of sequence similarity varied. Some regions had fewer mismatches between chicken and shorebirds than others. To identify conserved flank regions we aligned shorebird and chicken microsatellite sequences for 33 sequences with an E-value of E-19 or lower using CLUSTAL W algorithm [64] with the default options implemented in MEGA 3.1 software [65]. For 24 *Charadriiformes* microsatellite loci we were able to design consensus primers with a maximum of three base mismatches per primer pair (sequences are provided in Supplementary material). Only one of the 24 consensus primer sets had a perfect match between the *Charadriiformes* and chicken sequence. Therefore we introduced binary degenerated bases into the primer sequence at mismatch positions which provided a consensus for both sequences. If degenerated bases were introduced and several suggested primer candidates had the minimal number of three or less base mismatches, we chose the candidate which had base mismatches closer to the primer's 5' end. If a 'pigtail' had been added to the reverse primer of the standard primer set for a locus, the same 'pigtail' was also added to the corresponding reverse primer of the consensus primer pair.

To obtain consensus primers we had to relax the conditions used for primer design (see above). $T_m$'s of consensus primers were lower than those of the standard primer sets. Therefore we tested all 24 consensus primers using a lower annealing temperature

gradient (50–62 °C). All other PCR conditions were kept the same as used with standard primer PCR amplifications. Consensus primers derived from a *Charadriiformes*-chicken alignment are labelled with the prefix "Gga-" (for *Gallus gallus*).

### Allelic variability and observed heterozygosities

Twenty-three of 27 loci that amplified successfully in all three species were assessed for their levels of heterozygosity and allelic variability (Tables 3 & 4). Primer sets of four loci were dropped. Primers for *BmaTATC353* and *BmaGACA456* had yielded in single amplified products when examined on an agarose gel, however when we examined polymorphism on the ABI3730 DNA Analyzer genotypes contained multiple peaks and the loci could not be reliably scored. Loci *K16* and *Calex-08* were found to be expressed sequence tag (EST) loci. EST loci were not included in the present study. Microsatellite markers have been previously obtained from EST databases [66,67] and their cross-species utility is described elsewhere [68] and DA Dawson, in preparation.

To characterise correlates of polymorphism we investigated two different measures. Allelic variability was the proportion of species in which we found two or more alleles for a given microsatellite locus tested in four presumably unrelated individuals for twelve species. Polymorphism tests were carried out only with a single primer pair (consensus or standard) for any given locus. If both, consensus and standard primers had amplified across all three test species we chose the set that produced the cleanest product. PCRs were performed with the same conditions as described for amplification, with the difference that the annealing temperature was a common temperature where the primer set had amplified in all three species. A fraction of the PCR product was loaded on an ABI 3730 Analyzer using dye set DS-30, filter set D and ROX size standard for allele size determination and the resulting genotypes were scored using GENEMAPPER 3.7 software. The twelve test species were chosen from different *Charadriiformes* branches to ensure phylogenetic independence (Kentish plover, whiskered auklet, ruff, collared pratincole (*Glareola pratincola*), brown skua (*Catharacta lonnbergi*), gull-billed tern (*Gelochelidon nilotica*), red-necked phalarope (*Phalaropus lobatus*), great snipe (*Gallinago media*), dunlin (*Calidris alpina*), oystercatcher (*Haematopus ostralegus*), avocet (*Recurvirostra avosetta*), greater sheathbill (*Chionis alba*)).

The second variable for polymorphism, observed heterozygosity, was determined in whiskered auklet, ruff and Kentish plover. Here we tested primers in a total of 16

individuals per species. Beside observed heterozygosity ($H_o$) we calculated expected heterozygosity ($H_e$) and estimated null allele frequency using the program CERVUS v2.0 [69]. We performed tests for linkage equilibrium and compliance to Hardy Weinberg equilibrium using the program GENEPOP v3.3 [70].

## Statistical analysis

Non-parametric tests were used to test whether locus conservation and primer design affected amplification success, allelic variability and observed heterozygosity.

To examine correlates of amplification success and polymorphism we designed several statistical models. Amplification success was a proportional response variable which could take the value 0/3 (no amplification in any species), 1/3 (amplification successful in one species), 2/3 (amplification successful in two species) and 3/3 (amplification successful in all three species). Associations of amplification success was examined statistically incorporating explanatory variables of the following three categories: i) characteristics of the microsatellite locus (repeat length; whether a microsatellite was interrupted or not; the type of the microsatellite motif, i.e. whether the repeated base unit was a di- or tetranucleotide; observed heterozygosity in species of isolation; and $\Delta T_mH$ DNA-DNA hybridisation value between source species and target species as a measure for genetic distance [40]), ii) characteristics of the homologous sequence in chicken (single hit or hitting at multiple locations, microsatellite retained or absent) and, iii) properties of the standard primers (number of mismatches between chicken and *Charadriiformes* sequence). For each locus only the amplification results of standard primers went into the analysis.

The response variables for polymorphism, allelic variability and observed heterozygosity were tested with the same explanatory variables as amplification success with the following deviation: the explanatory variable '$\Delta T_mH$ DNA-DNA hybridisation value' was dropped for the analysis of allelic variability since we tested for polymorphism over a range of species.

To find correlates for amplification and allelic variability we constructed two GLMs with appropriate error structure including all explanatory variables and two-way interactions. GLMs were then simplified based on Akaike information criterion (AIC, [71,72]). Model simplification was performed in rounds, removing the highest non-

significant parameter at the beginning of each round until the minimal AIC value was reached. The final models contained only explanatory variables with *P*-values smaller than 0.1. Each microsatellite locus was considered as unit of analysis.

For observed heterozygosity we used a Mixed effect model (GLMM) with the same explanatory variables as for amplification success (see above) acting as fixed effects. Target species and microsatellite locus were included into the model as nested random effects (target species | locus (target species)). GLMMs were simplified by removing non-significant parameters hierarchically starting with high-order terms to minimise model deviance. Model simplification was continued until the current and predecessor model deviated significantly from each other examined by a F-test. The final models contained only explanatory variables with *P*-values smaller than 0.1.

Statistical analyses were carried out using R software version 2.4.1 [73]. All presented tests are two-tailed.

## Authors' contribution

CK planned and conducted the bioinformatic, laboratory and statistical analyses. DAD and TAB initiated the study, planned and supervised the bioinformatic and laboratory work. TS advised on the statistical analyses. All authors were involved in writing the manuscript. All authors read and approved the final manuscript.

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**Table 1. Characteristics of conserved and anonymous *Charadriiformes* microsatellite loci.**

| Locus | EMBL Accession Number | E-Value of hit at chicken genome | | Attributes of hit* | Gga† | Start position (bp) | Repeat in source species | Repeat in chicken‡ | Source species of locus | | Reference of locus source sequence |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | forward | reverse | | | | | | | | |
| *a) both flanks matching chicken* | | | | | | | | | | | |
| 54F2 | AM600679 | 2.00E-52 | 2.00E-52 | multiple hits | | | GT | na | oystercatcher | *Haematopus ostralegus* | 54 |
| Apy03 | AJ391211 | 6.10E-27 | 6.10E-27 | unique hit | 3 | 84,503,161 | GTTA | not present | whiskered auklet | *Aethia pygmaea* | 14 |
| Apy07 | AJ391219 | 2.70E-54 | 2.70E-54 | unique hit | 3 | 77,863,320 | GATA | GA | whiskered auklet | *Aethia pygmaea* | 14 |
| Apy09 | AJ391222 | 1.00E-26 | 1.00E-26 | unique hit | Z | 22,453,535 | GATA | GATA | whiskered auklet | *Aethia pygmaea* | 14 |
| Apy10 | AJ391223 | 4.00E-16 | 4.00E-16 | unique hit | 5 | 38,015,770 | TAGT | not present | whiskered auklet | *Aethia pygmaea* | 14 |
| BmaAAAC336 | DQ173162 | 6.40E-14 | 6.40E-14 | unique hit | 2 | 43,160,754 | (GT)n(AAAC)n | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaAAAG043 | DQ173163 | 7.70E-11 | 7.70E-11 | unique hit | 1 | 44,172,746 | (AAAG)n (GA)n | (AAAG)n (GA)n | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaAAAG433 | DQ173164 | 2.00E-16 | 2.00E-16 | multiple hits | | | AAAG | na | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaAGGT503 | DQ173166 | 1.00E-55 | 1.00E-55 | multiple hits | | | AGGT | na | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaCCAT443 | DQ173170 | 1.10E-16 | 1.10E-16 | unique hit | Un | 9,291,164 | (CCAT) | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaGACA456 | DQ173172 | 9.50E-53 | 9.50E-53 | unique hit | 2 | 130,207,507 | (GATA)n(GACA)n | AT | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaGATA365 | DQ173173 | 5.10E-20 | 5.10E-20 | unique hit | 1 | 45,497,185 | GATA | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaGTTT534 | DQ173183 | 5.80E-11 | 5.80E-11 | unique hit | 15 | 8,352,630 | GTTT | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaTATC353 | DQ173184 | 5.50E-20 | 5.50E-20 | unique hit | Z | 35,392,869 | (GTAT)n(CTAT)n | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaTATC356 | DQ173185 | 2.90E-18 | 2.90E-18 | unique hit | 11 | 16,879,295 | (GGTA)n (GATA)n | (GT)n(AT)n | marbled murrelet | *Brachyramphus marmoratus* | 74 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BmaTATC371 | DQ173186 | 1.20E-21 | 1.20E-21 | unique hit | 7 | 26,287,773 | GATA | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaTATC453 | DQ173188 | 4.20E-72 | 4.20E-72 | unique hit | 1 | 92,432,225 | GATA | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaTGAA523 | DQ173191 | 9.20E-36 | 9.20E-36 | unique hit | 1 | 55,778,757 | GAAT | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| Calex-01 | AM072445-7 | 1.20E-29 | 1.20E-29 | unique hit | 1 | 48,120,909 | GT | GT | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-04 | AM072450 | 3.20E-29 | 3.20E-29 | unique hit | 2 | 25,373,689 | GT | AAAT | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-05 | AM072453 | 7.70E-23 | 7.70E-23 | unique hit | 2 | 35,085,671 | GT | GT | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-07 | AM072455 | 3.70E-07 | 3.70E-07 | unique hit | 2 | 96,973,015 | GT | GT | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-08 | AM072456 | 3.80E-45 | 3.80E-45 | unique hit | 2 | 123,963,826 | GT | GT | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-14 | AM072462-3 | 2.80E-20 | 2.80E-20 | unique hit | 14 | 7,445,274 | GT | GT | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-18 | AM072468 | 2.40E-13 | 2.40E-13 | unique hit | 17 | 2,510,756 | GT | GT | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-20 | AM072470 | 1.80E-22 | 1.80E-22 | multiple hits | | | GT | na | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-26 | AM072478 | 6.60E-18 | 6.60E-18 | unique hit | Z | 64,181,833 | GT | not present | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-28 | AM072481 | 1.20E-14 | 1.20E-14 | multiple hits | | | GT | na | Kentish plover | *Charadrius alexandrinus* | 37 |
| Cmms3 | AB205034 | 2.00E-62 | 2.00E-62 | unique hit | 2 | 68,037,020 | GA | GA | rhinocerus auklet | *Cerorhinca monocerata* | 75 |
| Cmms9 | AB205036 | 1.90E-80 | 1.90E-80 | unique hit | 2 | 124,890,672 | GT | GT | rhinocerus auklet | *Cerorhinca monocerata* | 75 |
| Cmms14 | AB205037 | 2.00E-61 | 2.00E-61 | unique hit | 2 | 146,662,775 | GT | GT | rhinocerus auklet | *Cerorhinca monocerata* | 75 |
| Cmms22 | AB205038 | 5.80E-35 | 5.80E-35 | unique hit | 1 | 99,439,162 | GT | not present | rhinocerus auklet | *Cerorhinca monocerata* | 75 |
| Cmms23 | AB205039 | 2.20E-28 | 2.20E-28 | unique hit | 2 | 29,713,279 | GT | GT | rhinocerus auklet | *Cerorhinca monocerata* | 75 |
| Cmms26 | AB205040 | 5.50E-47 | 5.50E-47 | unique hit | 1 | 121,285,430 | GT | not present | rhinocerus auklet | *Cerorhinca monocerata* | 75 |
| SNIPE B2 | AY363298 | 4.20E-52 | 4.20E-52 | unique hit | 1 | 85,466,778 | GATA | (GT)n(AT)n | great snipe | *Gallinago media* | 76 |
| SNIPE 3 | AY363299 | 1.70E-20 | 1.70E-20 | multiple hits | | | GATA | na | great snipe | *Gallinago media* | 76 |
| K16 | AY083597 | 1.40E-31 | 1.40E-31 | unique hit | 11 | 6,461,653 | (GT)n(AT)n(GA)n | (GT)n(AT)n(GA)n | black-legged kittiwake | *Rissa tridactyla* | 77 |
| K56[1] | AY083600 | 5.90E-41 | 5.90E-41 | multiple hits | | | GT | GT | black-legged kittiwake | *Rissa tridactyla* | 77 |
| K71 | AY083602 | 1.70E-14 | 1.70E-14 | unique hit | 2 | 3,512,803 | GT | GT | black-legged kittiwake | *Rissa tridactyla* | 77 |
| LarsZAP14 | DQ251033 | 1.20E-16 | 1.20E-16 | unique hit | 1 | 24,899,043 | GT | GT | Herring gull | *Larus argentatus* | 78 |
| LarsZAP26/K32 | DQ251035 | 8.90E-12 | 8.90E-12 | unique hit | 3 | 1,545,186 | GT | GT | Herring gull | *Larus argentatus* | 78 |
| Mopl3 | DQ515758 | 1.30E-43 | 1.30E-43 | unique hit | Z | 40,051,800 | (CA)nGA(CA)n | (CA)nGA(CA)n | mountain plover | *Charadrius montanus* | 79 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mopl6 | DQ515760 | 3.10E-06 | 1.60E-36 | unique hit | 10 | 6,348,754 | (AT)n(GT)n | (AT)n(GT)n | mountain plover | *Charadrius montanus* | 79 |
| Mopl8 | DQ515761 | 1.90E-31 | 1.90E-31 | unique hit | 5 | 20,606,141 | GT | not present | mountain plover | *Charadrius montanus* | 79 |
| Mopl15 | DQ515764 | 1.90E-34 | 1.90E-34 | unique hit | 1 | 150,800,494 | GT | GT | mountain plover | *Charadrius montanus* | 79 |
| Mopl17 | DQ515765 | 1.80E-44 | 1.80E-44 | unique hit | 2 | 44,954,005 | GT | GT | mountain plover | *Charadrius montanus* | 79 |
| Mopl18 | DQ515766 | 6.10E-110 | 6.10E-110 | unique hit | 6 | 3,207,165 | (AT)n (CT)n | (AT)n (CT)n | mountain plover | *Charadrius montanus* | 79 |
| Mopl19 | DQ515767 | 2.40E-18 | 2.40E-18 | unique hit | 22 | 2,760,188 | GT | GT | mountain plover | *Charadrius montanus* | 79 |
| Mopl21 | DQ515768 | 3.30E-92 | 3.30E-92 | unique hit | 3 | 30,023,348 | GT | GT | mountain plover | *Charadrius montanus* | 79 |
| Mopl22 | DQ515769 | 4.20E-79 | 4.20E-79 | unique hit | 1 | 41,380,324 | GT | not present | mountain plover | *Charadrius montanus* | 79 |
| Mopl26 | DQ515771 | 3.40E-87 | 3.40E-87 | unique hit | 1 | 160,108,010 | GT | GT | mountain plover | *Charadrius montanus* | 79 |
| Pgt83 | AY198173 | 1.70E-33 | 1.70E-33 | unique hit | 12 | 11,734,131 | GT | GT | red knot | *Calidris canutus* | Buehler and Baker unpubl |
| Rbg18 | AY091847 | 1.40E-42 | 1.40E-42 | unique hit | 9 | 15,167,245 | GT | GT | red-billed gull | *Larus novaehollandiae* | 80 |
| Rbg27[2] | AY091851 | 1.20E-31 | 1.20E-31 | unique hit | 3 | 109,000,631 | GT | GT | red-billed gull | *Larus novaehollandiae* | 80 |
| Rbg29[3] | AY091853 | 6.80E-46 | 6.80E-46 | unique hit | 2 | 90,692,994 | GT | AT | red-billed gull | *Larus novaehollandiae* | 80 |
| Ruff50 | AF473576 | 1.80E-14 | 1.80E-14 | unique hit | 18 | 7,536,672 | GT | GT | ruff | *Philomachus pugnax* | 81 |
| ULO12A22 | AF195181 | 3.30E-07 | 3.30E-07 | unique hit | 2 | 83,079,429 | GA | not present | common murre | *Uria aalge* | 82 |
| *b) one flank matching chicken* | | | | | | | | | | | |
| 44B7 | AM600678 | 1.90E-12 | na | unique hit | 17 | 3,163,238 | GTTTT | not present | oystercatcher | *Haematopus ostralegus* | 54 |
| Apy16 | AJ391207 | 3.00E-12 | na | multiple hits | | | GT | na | whiskered auklet | *Aethia pygmaea* | 14 |
| BmaATAC370 | DQ173167 | 6.10E-41 | na | unique hit | 5 | 39,802,901 | (GTAT)n(GT)n | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaGATA439 | DQ173174 | 4.30E-20 | na | unique hit | 1 | 89,323,876 | (GATA)n (GACA)n | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaGATA464 | DQ173175 | na | 1.20E-28 | unique hit | Un | 13,162,817 | (GATA)n (GACA)n (CATA)n | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaGTTT515 | DQ173182 | na | 5.00E-14 | unique hit | 4 | 8,352,630 | GTTT | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaTATC444 | DQ173187 | 3.40E-39 | na | unique hit | 1 | 23,587,979 | (GATA)n(CATA)n | not present | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| Calex-02 | AM072448 | na | 2.40E-13 | unique hit | 1 | 166,776,604 | GT | GT | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-12 | AM072460 | 1.3E-80 | na | unique hit | 12 | 10,780,544 | (GT)n(GA)n | (GT)n(GA)n | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-17 | AM072467 | na | 1.30E-28 | unique hit | 17 | 6,818,576 | GT | not present | Kentish plover | *Charadrius alexandrinus* | 37 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Calex-19 | AM072469 | 4.40E-35 | na | unique hit | 20 | 11,647,572 | GT | not present | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-22 | AM072472-3 | na | 3.90E-15 | unique hit | 3 | 39,785,582 | GT | not present | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-23 | AM072474-5 | na | 1.70E-39 | unique hit | 1 | 197,982,722 | GT | not present | Kentish plover | *Charadrius alexandrinus* | 37 |
| Cmms2 | AB205033 | 1.30E-35 | na | unique hit | 3 | 64,727,099 | (CT)n(TG)n | not present | rhinocerus auklet | *Cerorhinca monocerata* | 75 |
| SNIPE B5 | AY363300 | na | 8.70E-25 | unique hit | 3 | 55,654,241 | GATA | GATA | great snipe | *Gallinago media* | 76 |
| SNIPE B20 | AY363302 | na | 5.10E-28 | multiple hits | | | GATA | na | great snipe | *Gallinago media* | 76 |
| Mopl2 | DQ515757 | 1.10E-55 | na | unique hit | 1 | 133,431,910 | CA | not present | mountain plover | *Charadrius montanus* | 79 |
| Mopl5 | DQ515759 | na | 3.10E-73 | unique hit | 4 | 87,425,385 | (CA)n(CT)n | not present | mountain plover | *Charadrius montanus* | 79 |
| Mopl13 | DQ515763 | na | 5.30E-121 | unique hit | 5 | 14,498,356 | GT | not present | mountain plover | *Charadrius montanus* | 79 |
| Mopl24 | DQ515770 | 4.70E-77 | na | unique hit | 1 | 124,293,234 | (CCAT)n(CCCA)n | not present | mountain plover | *Charadrius montanus* | 79 |
| Rbg13 | AY091848 | 7.80E-15 | na | multiple hits | | | GT | na | red-billed gull | *Larus novaehollandiae* | 80 |
| Rbg20 | AY091849 | na | 2.50E-13 | multiple hits | | | GT | na | red-billed gull | *Larus novaehollandiae* | 80 |
| Rbg28 | AY091850 | 2.80E-16 | na | unique hit | 11 | 8,499,868 | GT | na | red-billed gull | *Larus novaehollandiae* | 80 |
| Rbg39 | AY091852 | na | 3.30E-16 | unique hit | 5 | 8,934,560 | GT | AT | red-billed gull | *Larus novaehollandiae* | 80 |
| *c) anonymous loci* | | | | | | | | | | | |
| 9E6 | | 5.40E-02 | na | multiple hits | | | (GTGC)n(GT)n | na | oystercatcher | *Haematopus ostralegus* | 54 |
| Apy02 | AJ391209 | na | 1.80E-07 | unique hit | | | GATA | na | whiskered auklet | *Aethia pygmaea* | 14 |
| BmaGGAT313 | DQ173178 | na | 4.60E-08 | unique hit | | | GGAT | na | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| BmaCA382 | DQ173192 | na | na | no hit | | | CA | na | marbled murrelet | *Brachyramphus marmoratus* | 74 |
| Calex-24 | AM072476 | na | 6.00E-04 | multiple hits | | | (GT)n(GC)n | na | Kentish plover | *Charadrius alexandrinus* | 37 |
| Calex-37 | AM072492-3 | 1.70E+00 | na | multiple hits | | | GT | na | Kentish plover | *Charadrius alexandrinus* | 37 |
| K31 | AY083598 | na | na | no hit | | | GT | na | black-legged kittiwake | *Rissa tridactyla* | 77 |
| K67 | AY083601 | 7.90E-07 | 7.90E-07 | unique hit | | | AT | na | black-legged kittiwake | *Rissa tridactyla* | 77 |
| LarsNX01 | DQ251028 | 1.10E-08 | na | unique hit | | | (AC)n(TG)n(AT)n | na | Herring gull | *Larus argentatus* | 78 |
| Sdaat46 | AY597043 | 3.40E+00 | na | multiple hits | | | AAT | na | Roseate tern | *Sterna dougalli* | 83 |

[*]'Unique hit' refers to sequences that hit only to a single homolog and those that hit more than one location but the second hit was at least five decimals higher than the first hit; 'multiple hits' include all sequences that had several hits and the difference between first and second hit was less than five decimals. Only unique hits were mapped to chicken chromosomal map. If a microsatellite sequence hit to several locations in the chicken genome only the hit with the smallest E-value is presented.

[†]Gga, name of the chicken chromosome to which the hit was assigned.

[‡]Microsatellite motif found at the chicken location. 'not present' if the microsatellite was not retained, 'na' if the microsatellite could not be assigned to a single location because of multiple matches.

[1]microsatellite homologous to locus LarsNX24 [78]

[2]microsatellite homologous to locus LarsZAP11 [78]

[3]microsatellite homologous to locus LarsZAP19 [78]

**Table 2. Generalised linear models for a) amplification success and b) polymorphism of conserved microsatellite loci.**

| **a) Amplification success**[1] | | | | | | **b) Polymorphism**[2] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Error *df* * | Residual deviance* | *B* | *t* | *P* | | Error *df* * | Residual deviance* | *B* | *t* | *P* |
| Maximum model | 39 | 57.29 | | | | Maximum model | 12 | 24.97 | | | |
| - Interactions | 49 | 62.32 | | | | - Microsatellite retained | 13 | 24.97 | | | |
| - Repeat length | 50 | 62.32 | | | | - Primer type[5] | 14 | 25.46 | | | |
| - Unique/multiple hits | 51 | 62.7 | | | | - $H_o$ in source species[6] | 17 | 26.91 | | | |
| - Microsatellite motif[3] | 52 | 63.55 | | | | - No of mismatches[7] | 18 | 27.6 | | | |
| - No of mismatches[4] | 53 | 67.65 | | | | - E-value | 19 | 30.15 | | | |
| Minimum adequate model | | | | | | Minimum adequate model | | | | | |
| E-value | 53 | 67.65 | -0.02 | -2.58 | 0.013 | Repeat length | 19 | 30.15 | 0.02 | 2.22 | 0.039 |
| | | | | | | Microsatellite motif[3] | 19 | 30.15 | -1 | -2.15 | 0.045 |
| | | | | | | Microsatellite interrupted[8] | 19 | 30.15 | -1.02 | -2.09 | 0.051 |

[*]model descriptives are given for models after explanatory variable removal

[1]based on tests in three *Charadriiformes* species Kentish plover *Charadrius alexandrinus*, whiskered auklet *Aethia pygmaea* and ruff *Philomachus pugnax*

[2]based on proportion of species in which a microsatellite locus was found to be polymorphic when tested in four individuals

[3]*Di* or *tetra* core repeat motif of microsatellite

[4]total number of mismatches between chicken and *Charadriiformes* sequence for both primer sequences combined

[5]Gga-consensus primer or standard primer design

[6]observed heterozygosity for source species given in original publication of the microsatellite sequence

[7]number of mismatches in primer sequence between *Charadriiformes* sequence and chicken homolog

[8]microsatellite sequence interrupted (imperfect) by non-motif base insertions or not (perfect)

**Table 3. Expected and observed allele sizes of conserved chicken-*Charadriiformes* microsatellite markers.** The markers were tested in 12 *Charadriiformes* species tested in four unrelated individuals from a single population.

| Locus | Exp. allele size (bp)* | Observed allele size (bp) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Charadri* | | | | *Lari* | | | | *Scolopaci* | | | |
| | | KPL | OYS | AVO | GSH | WAU | CPR | BSK | GBT | RUF | RNP | GSN | DUN |
| *a) standard primer design* | | | | | | | | | | | | | |
| BmaTATC371 | 200 | 144, 152, 156 | 144, 146 | 149, 153, 157, 161, 165 | 152 | 158, 171, 173, 176, 180, 181, 190 | 173, 185 | 178, 182 | 173, 177, 185 | 140 | 136 | failed | 136 |
| Cmms 9 | 252 | 241 | 241 | 241 | 245 | 252, 256, 258, 262 | 252 | 255 | 250 | 246 | 244 | failed | 243 |
| Cmms 26 | 313 | 320 | 318 | 319 | 339 | 304, 308, 310 | 320, 327, 329, 338 | 304 | 310 | 316, 324 | 324, 326 | 326 | 312 |
| Mopl 6 | 281 | 290, 292, 294 | 274 | 274 | 273 | 278 | failed | 283 | 267 | 301, 303, 305 | 283, 287, 291, 303 | 277, 293, 295 | 284, 288, 290, 292 |
| Mopl 18 | 141 | 139 | 137 | 137 | 152 | 136 | 129 | 136 | 128 | 137 | failed | 136, 140 | 136 |
| Mopl 21 | 230 | 315, 321 | 317 | 321 | 320 | 315, 322 | 322 | 322 | 328, 330, 334 | 314, 320 | 316, 318 | 322 | 309, 311, 313 |
| LarsZAP26/K32 | 125 | 100, 102 | 98 | 97 | 94 | 106, 108, 133 | failed | 120 | 122, 124, 126, 134 | 106 | 99 | 105 | 106, 108 |
| *b) consensus primer design* | | | | | | | | | | | | | |
| 54F2 | 207 | 215, 217 | 206, 208 | 216 | 189 | 220 | 217, 221 | 228 | 219 | 212 | 212 | 218, 226 | 212 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Apy07 | 191 | 146 | 146 | 144 | 146 | 170, 190, 194, 198, 202, 206 | 157 | 154 | 154 | 145 | 145 | 145 | 145 |
| BmaAGGT503 | 255 | 249 | 248 | 249 | 247 | 251, 252, 253, 254 | 255 | 249 | 249 | 257 | 258 | 263 | 256 |
| BmaTATC453 | 294 | 239 | 239 | 239 | 239 | 239 | 236 | 239 | 239, 241 | 239 | 239 | 237, 239, 241 | 239 |
| BmaTGAA523 | 120 | 71 | 73 | 73 | 74 | 77 | 74 | 78 | 78 | 71 | 73 | 74 | 73 |
| Calex-01 | 221 | 201, 209, 211, 213, 221, 227 | 197 | 200 | 197 | 201, 203 | 224 | 201 | 233, 235, 239, 244 | 207 | 197, 201 | 211 | 207 |
| Calex-05 | 197 | 179, 181, 183 | 173 | 171 | 168 | 173 | 173, 175 | 173 | 177, 179 | 173 | 171, 173 | 171 | 173, 175 |
| Cmms3 | 146 | 95 | 101 | 95 | 97 | 102, 106, 108, 110 | 93, 95, 97, 99, 101, 103 | 105 | 108 | 95 | 95 | 95 | 95 |
| SNIPE B2 | 212 | 170, 172 | 183 | failed | 172 | 164 | 165 | 164 | 165 | 176, 180, 184, 196, 200 | 164, 168, 172, 184, 192, 196 | 188, 196, 200, 204, 208, 212 | 188 |
| Mopl 15 | 170 | 174, 182 | 160 | failed | 161 | 182 | 178 | 172, 182 | 178 | 172 | 172 | 173 | 172 |
| Mopl 22 | 404 | 394, 404 | 388 | 404 | 394 | 402 | 389 | 406 | 393 | 401 | 404 | 394, 404 | 412 |
| Mopl 26 | 195 | 188, 190, 194, 200, 212 | 191 | 192, 194, 195 | 191 | 192, 193, 194 | 189, 199 | 188 | 185 | 184 | 174 | 184 | 185 |
| Pgt 83 | 151 | 132 | 147 | 147 | 147 | 142 | 147 | 142 | 142 | 149 | 142, 144, 161 | 141, 143 | 142, 150, 156, 160 |
| Rbg 18 | 270 | 259, 263, 265, 267, 269, 275, 277 | 262, 264 | 261, 265 | 268 | 260, 266, 272 | failed | 268, 271 | 266 | 259, 261 | 265, 267 | 259 | 256, 260 |
| Rbg 27 | 194 | 184 | 184 | 186 | 182 | 182 | 182 | 186, 188 | 182, 190, 196 | 184 | 186 | 186, 188 | 184 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rbg 29 | 129 | 127 | 125 | 125 | 127 | 116, 118, 126, 132, 136, 146 | 118, 120, 122 | 127 | 122, 132, 134, 142 | 127 | 127 | 120 | failed |

*expected product size in source species based on the sequence of individual originally cloned

KPL Kentish plover (*Charadrius alexandrinus*), OYS oystercatcher (*Haematopus ostralegus*), AVO avocet (*Recurvirostra avosetta*), GSH greater sheathbill (*Chionis alba*), WAU whiskered auklet (*Aethia pygmaea*), CPR collared pratincole (*Glareola pratincola*), BSK brown skua (*Catharacta lonnbergi*), GBT gull-billed tern (*Gelochelidon nilotica*), RUF ruff (*Philomachus pugnax*), RNP red-necked phalarope (*Phalaropus lobatus*), GSN great snipe (*Gallinago media*), DUN dunlin (*Calidris alpina*)

**Table 4. Observed allele sizes, heterozygosities and estimated frequency of null alleles of conserved microsatellite loci.** All primers were tested in 16 unrelated individuals from a single population in Kentish plover *Charadrius alexandrinus*, whiskered auklet *Aethia pygmaea* and ruff *Philomachus pugnax*. Populations with significant deviation from Hardy-Weinberg equilibrium are presented in bold.

| Locus | *Charadri*: Kentish plover | | | | | | | *Lari*: whiskered auklet | | | | | | | *Scolopaci*: ruff | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *N* | k | Allele size (bp) | $H_o$ | $H_e$ | Est. null alleles* | $p_{HW}$[1] | *N* | k | Allele size (bp) | $H_o$ | $H_e$ | Est. null alleles* | $p_{HW}$[1] | *N* | k | Allele size (bp) | $H_o$ | $H_e$ | Est. null alleles* | $p_{HW}$[1] |
| a) standard primer | | | | | | | | | | | | | | | | | | | | | |
| BmaTATC371 | 16 | 6 | 144, 148, 150, 152, 154, 156 | 0.75 | 0.62 | -0.12 | 0.45 | 16 | 12 | 158, 161, 169, 171, 173, 174, 176, 177, 178, 180, 181, 190 | 0.88 | 0.88 | -0.01 | 0.53 | 16 | 1 | 140 | 0 | 0 | 0 | na |
| Mopl 18 | 16 | 1 | 139 | 0 | 0 | 0 | na | 16 | 2 | 128, 136 | 0.06 | 0.06 | -0.01 | na | 16 | 1 | 137 | 0 | 0 | 0 | na |
| Mopl 21 | 15 | 2 | 315, 321 | 0.47 | 0.48 | -0.02 | 1 | 16 | 2 | 315, 322 | 0.13 | 0.12 | -0.02 | 1 | 16 | 3 | 308, 314, 320 | 0.69 | 0.54 | -0.14 | 0.31 |
| Mopl 6 | 16 | 4 | 290, 292, 294, 296 | 0.81 | 0.70 | -0.09 | 0.94 | 15 | 1 | 278 | 0 | 0 | 0 | na | 16 | 5 | 299, 301, 303, 305, 307 | 0.44 | 0.38 | -0.11 | 1 |
| Cmms 26 | 16 | 1 | 320 | 0 | 0 | 0 | na | 14 | 5 | 304, 306, 308, 310, 312 | 0.79 | 0.75 | -0.05 | 0.92 | 8 | 2 | 316, 324 | 0.38 | 0.33 | 0 | 1 |
| Cmms 9 | 16 | 2 | 239, 241 | 0.13 | 0.12 | -0.02 | 1 | 15 | 8 | 250, 252, 254, 256, 258, 260, 262, 268 | 0.87 | 0.75 | -0.11 | 0.94 | 14 | 1 | 246 | 0 | 0 | 0 | na |
| LarsZAP26/K32 | 16 | 2 | 100, 102 | 0.38 | 0.39 | 0 | 1 | 12 | 10 | **101, 106, 108, 117, 119, 123, 125, 129, 130, 133** | **0.58** | **0.90** | **0.20** | **0.01** | 15 | 1 | 106 | 0 | 0 | 0 | na |
| b) consensus primer | | | | | | | | | | | | | | | | | | | | | |
| 54F2 | **14** | **2** | **215, 217** | **0.14** | **0.42** | **0.48** | **0.03** | 16 | 1 | 220 | 0 | 0 | 0 | na | 16 | 1 | 212 | 0 | 0 | 0 | na |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Apy07 | 16 | 1 | 146 | 0 | 0 | 0 | na | 16 | 8 | 170, 182, 186, 190, 194, 198, 202, 206 | 0.75 | 0.78 | 0.01 | 0.80 | 16 | 1 | 145 | 0 | 0 | 0 | na |
| BmaAGGT503 | 16 | 1 | 249 | 0 | 0 | 0 | na | 16 | 4 | 251, 252, 253, 254 | 0.69 | 0.72 | 0.02 | 0.23 | 16 | 1 | 257 | 0 | 0 | 0 | na |
| BmaTATC453 | 16 | 2 | 235, 239 | 0.06 | 0.06 | -0.01 | na | 16 | 1 | 239 | 0 | 0 | 0 | na | 16 | 2 | 239, 241 | 0.13 | 0.12 | -0.02 | 1 |
| BmaTGAA523 | 16 | 1 | 71 | 0 | 0 | 0 | na | 16 | 2 | 69, 77 | 0.06 | 0.06 | -0.01 | na | 16 | 1 | 71 | 0 | 0 | 0 | na |
| Calex-01 | 13 | 11 | 201, 205, 209, 211, 213, 215, 217, 219, 221, 223,227 | 1 | 0.91 | -0.07 | 0.32 | 16 | 3 | **199, 201, 203** | **0.19** | **0.54** | **0.52** | **<0.001** | 16 | 2 | 207, 209 | 0.06 | 0.06 | -0.01 | na |
| Calex-05 | 14 | 6 | 173, 179, 180, 181, 183, 184 | 0.64 | 0.71 | 0.03 | 0.60 | 16 | 2 | 173, 175 | 0.38 | 0.32 | -0.10 | 1 | 16 | 1 | 173 | 0 | 0 | 0 | na |
| Mopl 15 | 16 | 6 | 170, 172, 174, 176, 180, 182 | 0.75 | 0.71 | 0.03 | 0.48 | 15 | 2 | 174, 182 | 0.07 | 0.07 | -0.01 | na | 15 | 1 | 172 | 0 | 0 | 0 | na |
| Mopl 22 | 13 | 2 | 394, 404 | 0.31 | 0.27 | -0.08 | 1 | 16 | 1 | 402 | 0 | 0 | 0 | na | 16 | 1 | 401 | 0 | 0 | 0 | na |
| Mopl 26 | 16 | 9 | 188, 190, 194, 196, 198, 200, 208, 210, 212 | 0.69 | 0.82 | 0.08 | 0.05 | 16 | 6 | 189, 190, 191, 192, 193, 194 | 0.75 | 0.73 | -0.04 | 0.80 | 16 | 1 | 184 | 0 | 0 | 0 | na |
| Cmms3 | 14 | 1 | 95 | 0 | 0 | 0 | na | 16 | 6 | **102, 106, 108, 110, 112, 114** | **0.81** | **0.78** | **-0.03** | **0.02** | 16 | 1 | 95 | 0 | 0 | 0 | na |
| SNIPE B2 | 16 | 2 | 170, 172 | 0.19 | 0.35 | 0.29 | 0.11 | 16 | 1 | 164 | 0 | 0 | 0 | na | 16 | 8 | 168, 172, 176, 180, 184, 192, 196, 200 | 0.75 | 0.80 | 0.03 | 0.32 |
| Pgt 83 | 14 | 1 | 132 | 0 | 0 | 0 | na | 16 | 1 | 142 | 0 | 0 | 0 | na | 16 | 2 | 149, 151 | 0.06 | 0.06 | -0.01 | na |
| Rbg 18 | 16 | 7 | 259, 263, 265, 267, 269, 275, 277 | 0.75 | 0.77 | 0.01 | 0.76 | 8 | 4 | 260, 264, 266, 272 | 0.38 | 0.66 | 0 | 0.08 | 16 | 3 | 257, 259, 261 | 0.13 | 0.12 | -0.02 | 1 |
| Rbg 27 | 16 | 1 | 184 | 0 | 0 | 0 | na | 16 | 2 | 180, 182 | 0.06 | 0.06 | -0.01 | na | 15 | 1 | 184 | 0 | 0 | 0 | na |

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rbg 29 | 15 | 1 | 127 | | 0 | 0 | 0 | na | 16 | 12 | **116, 118, 120, 124, 126, 128, 130, 132, 136, 138, 144, 146** | **0.69** | **0.90** | **0.13** | **<0.01** | 14 | 1 | 127 | | 0 | 0 | 0 | na |

*N,* number of individuals amplified in polymorphism test; k, number of alleles found in test sample; $H_o$, observed heterozygosity $H_e$, expected heterozygosity; [*]estimate of null alleles using CERVUS v. 2.0; [1]probability that locus is in Hardy-Weinberg equilibrium estimated by GENEPOP v 3.3

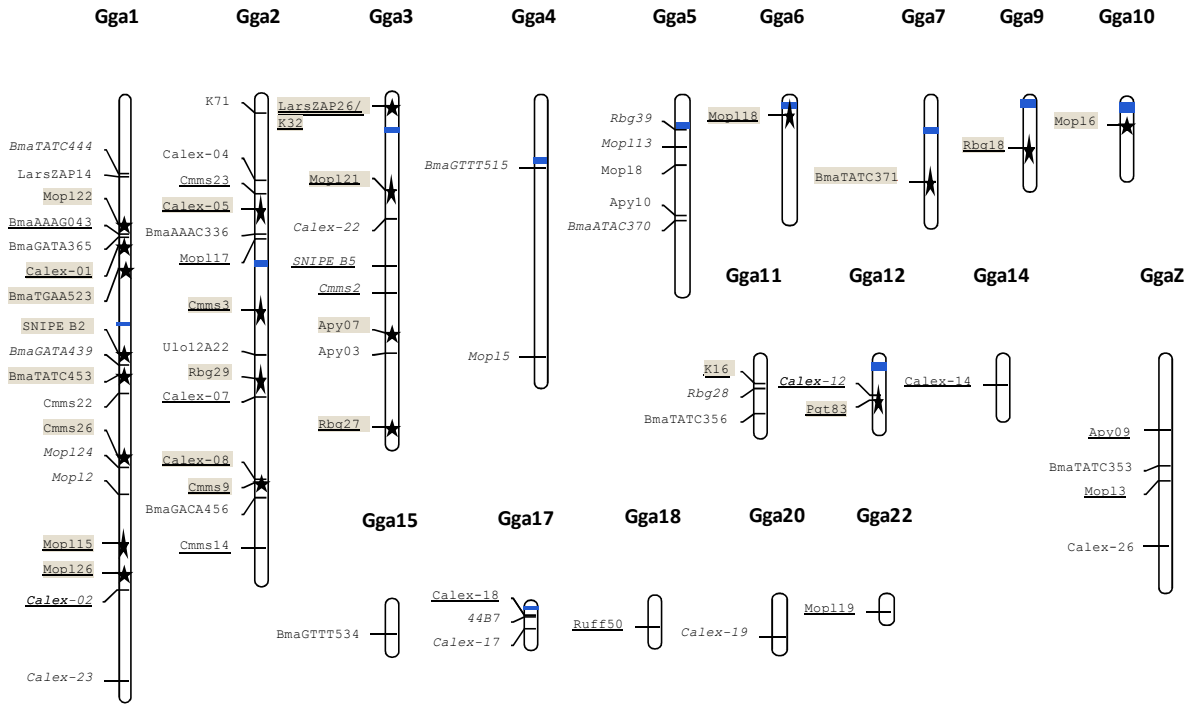


**Figure 1. Chromosome map of the chicken displaying the genomic locations of 68 conserved microsatellite homologs that were isolated in different *Charadriiformes* species.** If the microsatellite motif was found to be retained in the chicken homolog the locus name is underlined. Microsatellite loci examined for polymorphism are marked by a star. Shaded loci represent the microsatellite loci that could be amplified in *Lari*, *Charadri* and *Scolopaci* with either standard or consensus primers. For loci shown in italics only one of the flanks (forward or reverse) was assigned to the map. Centromere locations that could be deduced by high GC content on the chicken map following [17] are highlighted in blue. Z location for the four microsatellite markers was confirmed by hemizygous segregation of genotypes in females for *Apy09*, *BmaTATC353, Mopl3* and *Calex-26.*

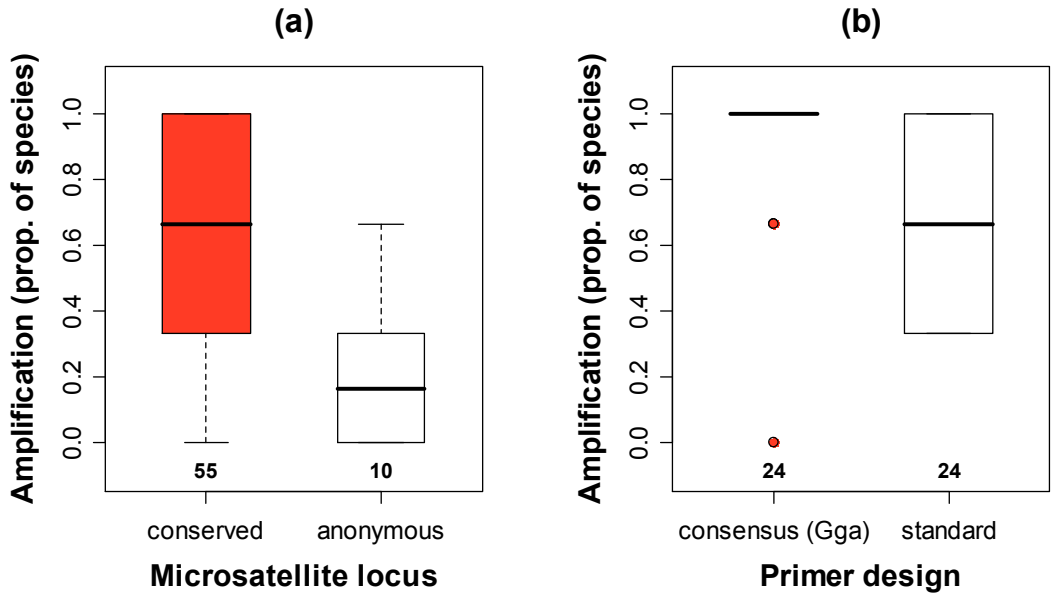


**Figure 2. Amplification success for conserved microsatellite loci and primer sets across the major *Charadriiformes* lineages.** Conserved microsatellite loci refer to the loci for which both flanking regions could be located to a homolog in the chicken genome. Anonymous sequences lacked matching flanks. Each *Charadriiformes* lineage was represented by one species: Kentish plover for *Charadri*, whiskered auklet for *Lari* and ruff for *Scolopaci*. (a) Amplification success for standard primers derived from both conserved microsatellite loci and anonymous microsatellite loci. (b) Amplification success of consensus and standard primers for conserved loci in which both primers were designed. Consensus primers were designed after alignment of chicken and *Charadriiformes* sequence and placed into highly preserved flanking regions between chicken and shorebird. Standard primers were designed using the shorebird sequence only without comparison to the chicken sequence homolog. Numbers in the bottom refer to (a) the number of microsatellite loci and (b) the number of primers that were tested in each group.

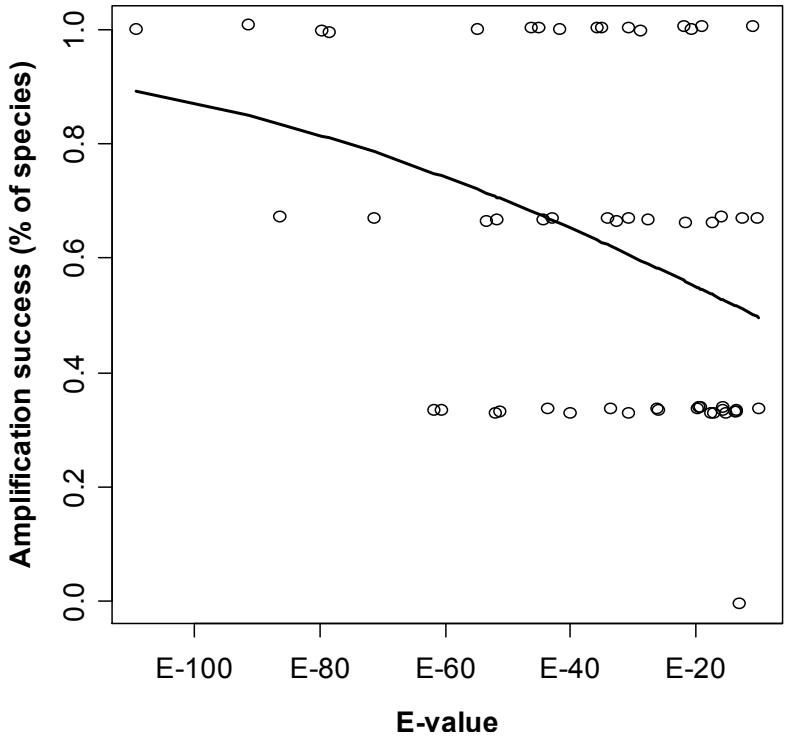


**Figure 3. Amplification success of conserved microsatellite loci in three *Charadriiformes*-species Kentish plover, whiskered auklet and ruff in relation to the E-value of the chicken-*Charadriiformes* hit.** Loci with both flanks matching the chicken sequence at the same chromosomal region of the chicken are considered. Smaller E-values indicate higher probability of identity. Open circles stand for a single microsatellite locus. The line represents predicted values derived from the statistical model (see text).

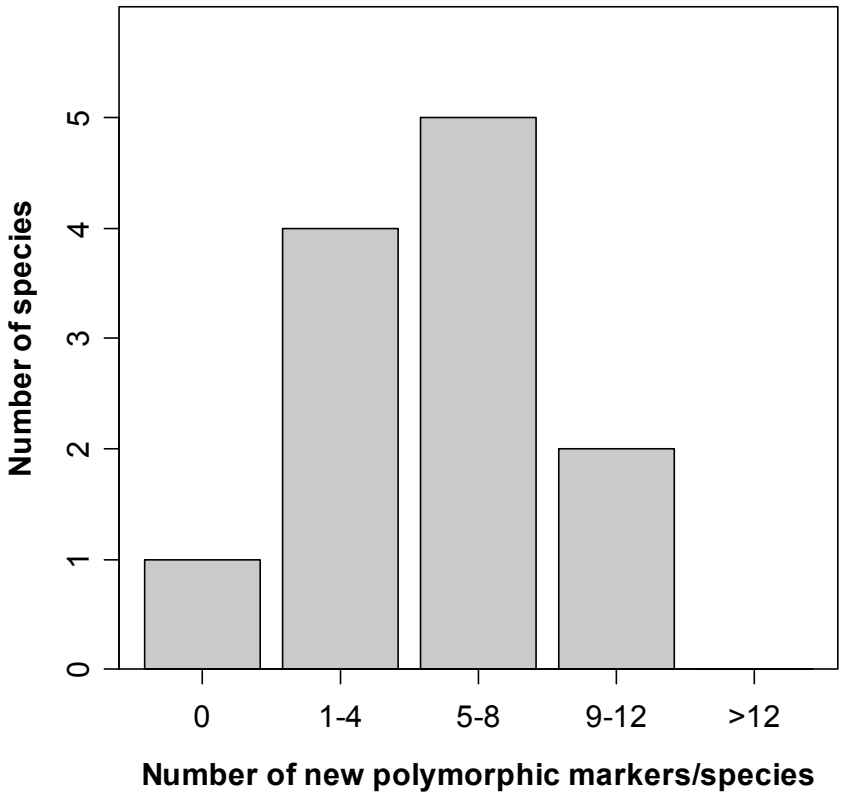


**Figure 4. Number of newly derived polymorphic microsatellite markers for 12 *Charadriiformes* species when tested in four unrelated individuals**. In total 24 conserved *Charadriiformes* microsatellite markers were tested. Data are only included if test and source species of the microsatellite marker were different.

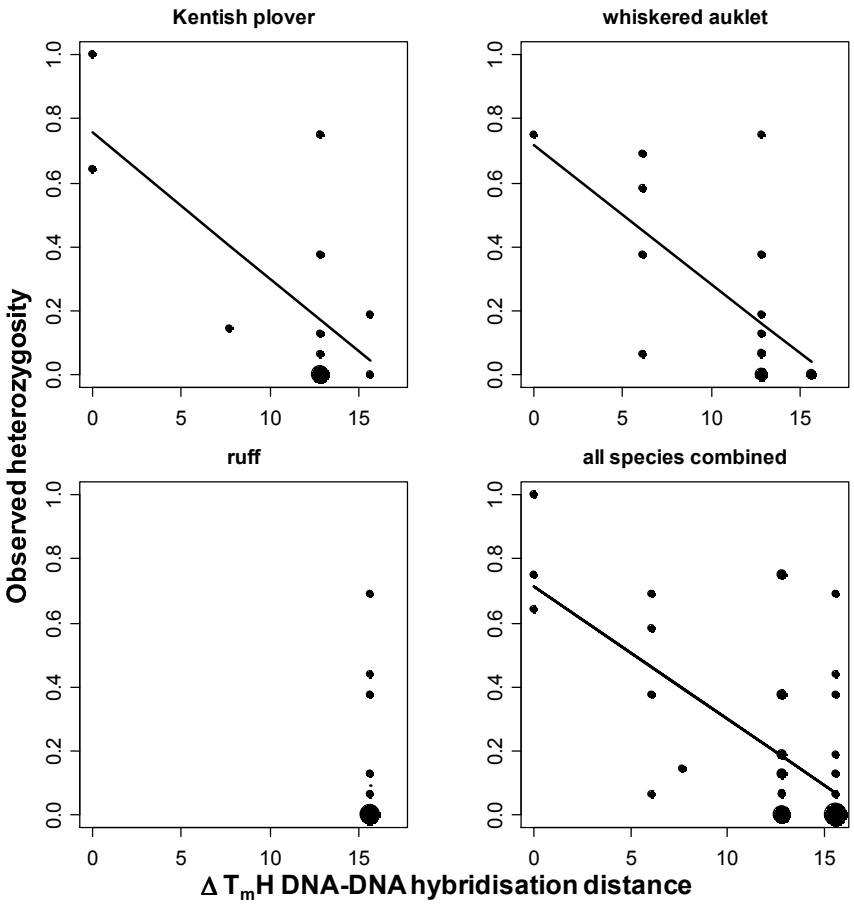


**Figure 5. Observed heterozygosity in relation to $\Delta T_m H$ DNA-DNA hybridisation distance between source and test species for 23 *Charadriiformes* microsatellite loci.** Size of circles is proportional to the number of data points at a given location. The trend line was drawn using predicted values from Generalised Linear Models for each of the three species separately and from predicted values from a Mixed effect model for all species combined including species and locus as hierarchical random factors.

## Supplementary Material

Primer sequences, EMBL accession number, amplification conditions and amplification results of all *Charadriiformes* microsatellite loci tested.

| Locus | EMBL Access No. | Primer type | Primer sequence 5' - 3' (pigtail underlined) | Ta in °C‡ | Conc. $MgCl_2$ in mM | Amplification Kentish plover | ruff | whiskered auklet |
|---|---|---|---|---|---|---|---|---|
| 54F2 | AM600679 | Standard | F: GTATTACTTTGTGCCCAGGGTTGTT | 54-64 | 2.0 | 1 | 1 | 0 |
| | | Standard | R: TAATTGCATTGCACATCAGCTCTAA | | | | | |
| Gga54F2 | AM600679 | Consensus | F: ACAGAGG**W**GGCTGC**R**TTG | 50-62 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: TTCAAATTGGCTGCAGGTTA | | | | | |
| Apy03 | AJ391211 | Standard | F: TTTCCTTTGATGTTCACTACAAACA | 54-58 | 2.0 | 0 | 0 | 1 |
| | | Standard | R: AATGGGAGAGGCACGATGT | | | | | |
| Apy07 | AJ391219 | Standard | F: ATCGCTCCCTGGCACATTC | 54-59 | 2.0 | 0 | 1 | 1 |
| | | Standard | R: GTTTCTTTCGTATGAAATAATCTGGGGCATC | | | | | |
| GgaApy07 | AJ391219 | Consensus | F: TG**Y**GGAAACATTTGGCAAGAAA | 50-62 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: GTTTCTTTGAAATAATCTGGGGCA**Y**CACA | | | | | |
| Apy09 | AJ391222 | Standard | F: GCTGACATGAGAGGTCACCA | 54-61 | 2.0 | 0 | 0 | 1 |
| | | Standard | R: CCGGATTAACCACTTTTACCTG | | | | | |
| Apy10 | AJ391223 | Standard | F: GCAGTTCTTGTTTAGCATGGC | 54-57 | 2.0 | 0 | 0 | 1 |
| | | Standard | R: GTTTCTTAGTGCAAGACCGAACCTCAG | | | | | |
| BmaAAAC336 | DQ173162 | Standard | F: GCGTTATGAAATAGCCTGCTGAA | 54-59 | 2.0 | 0 | 0 | 0 |
| | | Standard | R: CAGCGATGAAATGCTGTGTGTAG | | | | | |
| BmaAAAG043 | DQ173163 | Standard | F: CCAAATTGACAGAAACAGTTAATCCA | 54-55 | 2.0 | 0 | 1 | 0 |
| | | Standard | R: TGTAATTACCAGTAAGCCACTGTCAGA | | | | | |
| BmaAAAG433 | DQ173164 | Standard | F: CAGAATCACCGCATTCAGAAGAT | 54-57 | 2.0 | 0 | 0 | 1 |
| | | Standard | R: ACTGAGAAATCCAAAGGCCAAAG | | | | | |
| BmaAGGT503 | DQ173166 | Standard | F: GAGGAATATTGTAGGAGGGAGAT | 54-65 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: TTTAAGTCTAATATTGGTCTCTCAGC | | | | | |
| GgaBmaAGGT503 | DQ173166 | Consensus | F: GCATGGGAGGAATATTGTAG | 50-60 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: **R**AGTCTAATATTGGTCTC**W**CA**R**C | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| BmaCCAT443 | DQ173170 | Standard | F: TGCCAGGCCATCTACTTTAATGA | 64-66 | 2.0 | 1 | 0 | 1 |
| | | Standard | R: ATAATAAGCTGGGCTGCCAACA | | | | | |
| BmaGACA456 | DQ173172 | Standard | F: AACTGGTCTCTTTGCTTGATGGA | 54-57 | 2.0 | 0 | 0 | 1 |
| | | Standard | R: TGCAGTGGGACAAGAAGGATAAG | | | | | |
| GgaBmaGACA456 | DQ173172 | Consensus | F: AAAAACTGGTCTCTTTGCTT | 51-53 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: ACAAGTGA**Y**AGGAAAAA**K**TGC | | | | | |
| BmaGATA365 | DQ173173 | Standard | F: TTATCTGTGGCAACACTGTCGAA | 54-65 | 2.0 | 0 | 0 | 1 |
| | | Standard | R: ATGATGCATAGCAACCAGCAGAT | | | | | |
| BmaGTTT534 | DQ173183 | Standard | F: CGAGTTCCTTGGAGGAAAGAGAT | 54-62 | 2.0 | 1 | 0 | 1 |
| | | Standard | R: CCATGGCTTTATATGGAATCACAA | | | | | |
| BmaTATC353 | DQ173184 | Standard | F: ATGCTCTGGACTGACTTGTGGTC | 54-55 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: AGACTATATAGCCCATTCCCACTTCC | | | | | |
| BmaTATC356 | DQ173185 | Standard | F: GTGGTCCACTGAGTTTAGCAGCA | 54-57 | 2.0 | 0 | 1 | 1 |
| | | Standard | R: TGAAGCATGGGACTCTGTAGTGG | | | | | |
| BmaTATC371 | DQ173186 | Standard | F: CAGTTTGGCTCTCCAAAGAAACA | 54-61 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: TCGATAGGCTTTAAATTCGAGTGAA | | | | | |
| BmaTATC453 | DQ173188 | Standard | F: ACCTGGCAGAATCACAGATGTTC | 54-55 | 2.0 | 0 | 1 | 1 |
| | | Standard | R: TCAGGAGCACCATGTATGTTTGA | | | | | |
| GgaBmaTATC453 | DQ173188 | Consensus | F: C**R**CATGTTTTGC**W**GTAGACAA | 50-60 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: TGTTGATGGAGTAACCAGGA | | | | | |
| BmaTGAA523 | DQ173191 | Standard | F: ATCGCTTCAGACATCCAGAGTTA | 54-64 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: CTAATGAACTAATGAGGGCGATG | | | | | |
| GgaBmaTGAA523 | DQ173191 | Consensus | F: TGAATCCAGTGGAA**Y**AAAACAACA | 50-62 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: TGAGGGC**R**ATGAAAGGAGAA | | | | | |
| Calex-01 | AM072445-7 | Standard | F: CTTCTCCATTGTTGTCACCTCCAGT | 54-66 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: GTTTCTTCTTGACTTGGCCTGAGGTTTAGGTT | | | | | |
| GgaCalex-01 | AM072445-7 | Consensus | F: CACCATGGAGATTGGTTCTGCTATG | 50-62 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: GTTTCTTAGCC**Y**TGACTTGGCCTGAGGTTTA | | | | | |
| Calex-04 | AM072452 | Standard | F: CAGGCAACAATCCCAGTCTTATC | 54-64 | 2.0 | 1 | 1 | 0 |
| | | Standard | R: TTTGACTTGACAAGCAGCTTCC | | | | | |
| Calex-05 | AM072453 | Standard | F: TCCAGCTGAAGTCTTCCGTGAAT | 54-64 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: GTTTCTTTCCACACCTGTTCGACAGTTCAATA | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GgaCalex-05 | AM072453 | Consensus | F: CACTCTATTTTTCCTC**Y**AGCTGAAGTCT | 50-62 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: GTTTCTTATAGAAGCCTGCTTTTGATGGAAGC | | | | | |
| Calex-08 | AM072456 | Standard | F: CCTGCTTCATTTCGCATAAACTGAC | 54-66 | 2.0 | 1 | 0 | 1 |
| | | Standard | R: GTTTCTTCTTCCATGGTAAATTGCGACTCTTG | | | | | |
| GgaCalex-08 | AM072456 | Consensus | F: TTA**M**AGAATTCTTTCACATGGTCTCT | 50-52 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: GTTTCTTCGGAATATTAAGTAGAGGCTTCCAT | | | | | |
| Calex-14 | AM072462-3 | Standard | F: TCAGTTTGGAGACATTTTCCTACTAAGCA | 54-64 | 2.0 | 1 | 0 | 0 |
| | | Standard | R: GTTTCTTACAGAGCCGTAAGGAATGTGCAGTA | | | | | |
| Calex-18 | AM072468 | Standard | F: GAAGAGGGCTTTGCTTGTAAT | 54-59 | 2.0 | 1 | 0 | 1 |
| | | Standard | R: GTTTCTTACCAGTGTAATGCACTCCTGT | | | | | |
| GgaCalex-18 | AM072468 | Consensus | F: TTGC**Y**TGTAATGAT**R**CACAC | 53-60 | 2.0 | 1 | 0 | 1 |
| | | Consensus | R: GTTTCTTCCAGTGTAATGCACTCCTGT | | | | | |
| Calex-20 | AM072470 | Standard | F: TGTTTCGCAGGCTAATTTGGTAGG | 54-66 | 2.0 | 1 | 0 | 1 |
| | | Standard | R: AGCCGTGGAGAGCTGATGTTG | | | | | |
| Calex-26 | AM072478 | Standard | F: AAGCAAATGAGCTGGGCTGTGT | 54-64 | 2.0 | 1 | 0 | 0 |
| | | Standard | R: GTTTCTTATGCGTGGGCAGGGAAGAT | | | | | |
| Calex-28 | AM072481 | Standard | F: CAGTTGCTGGCACCTGGACA | 61-66 | 2.0 | 1 | 0 | 0 |
| | | Standard | R: GTTTCTTCGGGACACATTACGGGATGC | | | | | |
| Cmms3 | AB205034 | Standard | F: TGCACTGCAGAAAGCAGATATGTTA | 54 | 2.0 | 0 | 0 | 1 |
| | | Standard | R: CGTAGCCACCTGTTACACCCTTTAT | | | | | |
| GgaCmms3 | AB205034 | Consensus | F: AAAAGGCCACC**Y**CCACCTA | 55-62 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: GCACGTAGCCACCTGTTAC**R**CC | | | | | |
| Cmms9 | AB205036 | Standard | F: CTGGTGGGAATGACTGAGATTTG | 54-62 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: ATTCTACATCAGTGTGGGCTGGA | | | | | |
| GgaCmms9 | AB205036 | Consensus | F: GACTGAGATTTGAGCTCTGG | 50-53 | 2.0 | 0 | 1 | 1 |
| | | Consensus | R: CAGGAGCTGTTGTCCAAAC | | | | | |
| Cmms14 | AB205037 | Standard | F: GTAATCTCTCATTGTGCGCTGCT | 54-62 | 2.0 | 0 | 0 | 1 |
| | | Standard | R: TCTCATTATGCACAGGTGTCAC | | | | | |
| GgaCmms14 | AB205037 | Consensus | F: CTGGCTGGTAA**C**CTC**G**CATTGT | 50-53 | 2.0 | 1 | 0 | 1 |
| | | Consensus | R: **K**TGGTAACGTGCTGATGAATG | | | | | |
| Cmms22 | AB205038 | Standard | F: GCTTTCTTCTGGAGCAGTGTGAA | 54-64 | 2.0 | 1 | 1 | 0 |
| | | Standard | R: GACTTATGGTCGCCATATTCTCATTC | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Cmms23 | AB205039 | Standard | F: ATCTGCTCACAGCTTCCAACAGT | 54 | 2.0 | 0 | 1 | 1 |
| | | Standard | R: TAGGCTCTCAAACGTCAACACG | | | | | |
| Cmms26 | AB205040 | Standard | F: TCACACCGGAGCTAGTCAAAGAG | 54-62 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: TGCAAATGGTTTCCGTTGTTTAG | | | | | |
| SNIPE-B2 | AY363298 | Standard | F: CTGTACTTGGGCATCTTCCAAGC | 54-56 | 2.0 | 0 | 1 | 0 |
| | | Standard | R: GCAGGATATGGAGGCACTTGAAAT | | | | | |
| GgaSNIPE-B2 | AY363298 | Consensus | F: ATCTTCCA**R**GCAAATAAATAA | 54-57 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: AA**Y**TTCACAGTGCAAGGA | | | | | |
| SNIPE-3 | AY363299 | Standard | F: GCCCAGAACCTAGAGTTTCCAATTA | 54-56 | 2.0 | 0 | 1 | 0 |
| | | Standard | R: TGATGGTCCACAGACTAAGGATGTT | | | | | |
| GgaSNIPE-3 | AY363299 | Consensus | F: AGACCCAGGACATCATCCAC | 54-64 | 2.0 | 0 | 0 | 0 |
| | | Consensus | R: GCT**S**AAAGTCATGGA**Y**TAAATGAA | | | | | |
| K16 | AY083597 | Standard | F: TGCAATTTGTACAACCAGATTT | 54-64 | 2.0 | 0 | 1 | 0 |
| | | Standard | R: TATACCAAGTACCTAATGCAACTGA | | | | | |
| GgaK16 | AY083597 | Consensus | F: AAGTTTTCCATATAAAACATCTCA | 50 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: **K**AACTTGAAAAGCTGCAAAA | | | | | |
| K56[1] | AY083600 | Standard | F: ACACTAATGCTTTCAGTGCTCAGCT | 54 | 2.0 | 1 | 0 | 0 |
| | | Standard | R: GGACTTTCTTTGGGTCTTGAAATCT | | | | | |
| K71 | AY083602 | Standard | F: ACCAGGCATTTCCTTCAGCTTAC | 54 | 2.0 | 1 | 0 | 0 |
| | | Standard | R: AAATCACCACCTTCAAACCTCTCAT | | | | | |
| LarsZAP14 | DQ251033 | Standard | F: CACAGAAATACAAACCTGGGAATTA | 54-62 | 2.0 | 0 | 1 | 0 |
| | | Standard | R: TTTGGAAACCCTGTTAAATCTTGTA | | | | | |
| LarsZAP26[2] | DQ251035 | Standard | F: CCAGCATTGCACGAGTGTTAAG | 55-57 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: GCCTGTCCTTGTCCTGCTCTT | | | | | |
| Mopl3 | DQ515758 | Standard | F: CATCACAAGTCCACTTTCAGATGC | 54-57 | 2.0 | 1 | 1 | 0 |
| | | Standard | R: TGCTACATGGAACCTTCATTGCT | | | | | |
| Mopl6 | DQ515760 | Standard | F: CAATTCAATGGCACTTCCTTCTAAA | 54-66 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: TCCTTGCCACTTCTGAACACTTATC | | | | | |
| Mopl8 | DQ515761 | Standard | F: TTTGTTCTGATATGGCTCTTCC | 54-55 | 2.0 | 1 | 1 | 0 |
| | | Standard | R: ATGGTAGTTGAATGTTGTCTTGAAC | | | | | |
| Mopl15 | DQ515764 | Standard | F: ACTCACAAAGGGCTAAGGCATAAAC | 54-64 | 2.0 | 1 | 0 | 0 |
| | | Standard | R: TAAACCGAGATGTTATTTGGGCTCT | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GgaMopl15 | DQ515764 | Consensus | F: GAAA**S**AAAATCCTTTA**YW**CTAGACC | 54-59 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: TGAAAACAGCACCTAACTCTG | | | | | |
| Mopl17 | DQ515765 | Standard | F: AACCAGTGCATCATCCTAATCACAA | 54-64 | 2.0 | 1 | 0 | 0 |
| | | Standard | R: CCCAAACTGCAGTCTCTCCACTAAT | | | | | |
| Mopl18 | DQ515766 | Standard | F: GATCTCTTAGGGCAGAGTTGCTGTA | 54-55 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: CAAACAATAGGGCTTACCCACATTA | | | | | |
| Mopl19 | DQ515767 | Standard | F: GAAAGGAGAAACTGCAAGAAAA | 54-56 | 2.0 | 1 | 0 | 0 |
| | | Standard | R: TACTTCTGTTTACTCTCCCCTGAA | | | | | |
| Mopl21 | DQ515768 | Standard | F: AACTTCATGCAATTAAGTAATCAGA | 54-55 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: CCAGATTTCCTATACAGGTAGAAAG | | | | | |
| GgaMopl21 | DQ515768 | Consensus | F: TCATGCAATTAAGTAATCAGAA | 50-60 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: TAAAGCTGCAAATCT**R**ACAA | | | | | |
| Mopl22 | DQ515769 | Standard | F: AGGTCAAATGTTTGTTGCAGAAGAG | 54-66 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: ACAGGATTGGTCCTTGCACTTTAAC | | | | | |
| GgaMopl22 | DQ515769 | Consensus | F: GGCA**Y**C**K**TAGAAATAGTCCACAGGA | 50-62 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: GATTGGT**Y**CTTGCACTTTAACAACT | | | | | |
| Mopl26 | DQ515771 | Standard | F: CCTGGTCATTAACAAACCAGATGAG | 54-61 | 2.0 | 0 | 1 | 1 |
| | | Standard | R: GGAATGGCATATTTACATGTTCTGG | | | | | |
| GgaMopl26 | DQ515771 | Consensus | F: CAGGAATATAGCTA**Y**CATGCTTAAC | 50-62 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: GGG**S**TTTGGTGGTTGAACT | | | | | |
| Pgt83 | AY198173 | Standard | F: GAAGCACAGGACTGATGTCCAGA | 56-65 | 2.0 | 0 | 1 | 1 |
| | | Standard | R: CTCCACCCTTGAATGCATTAGAACT | | | | | |
| GgaPgt83 | AY198173 | Consensus | F: ACAGGACTGATGTCCAGAG | 54-59 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: TTTAAAA**S**AGCTTCTCTCCAG | | | | | |
| Rbg18 | AY091847 | Standard | F: TGTTCTGAAAGGGCTGCTCATAGTA | 54-66 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: GCATACCTTGCAAGTAGCATCATGT | | | | | |
| GgaRbg18 | AY091847 | Consensus | F: AA**R**TTCA**K**AAATCTGTTCTGAAAGG | 50-60 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: TTCCAACTGAGCCCTTGAC | | | | | |
| Rbg27[3] | AY091851 | Standard | F: TAAGCTTGCAGGCAATAATCTTGAG | 54-63 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: AACTTTCCCTTCCAAGAAATCACAG | | | | | |
| GgaRbg27[3] | AY091851 | Consensus | F: TG**R**CAGGATTGGTCTTGAAAA | 50-60 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: CCCT**Y**CCAAGAA**R**TCACAGTGAAA | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Rbg29[4] | AY091853 | Standard | F: TGGACTCAGTGCCCTCTTCC | 54-59 | 2.0 | 1 | 1 | 1 |
| | | Standard | R: AGTAACTGTATTCCAGGGCAGAAGC | | | | | |
| GgaRbg29[4] | AY091853 | Consensus | F: CCTAGCTTTTTGGACTCAGTG | 50-59 | 2.0 | 1 | 1 | 1 |
| | | Consensus | R: AATAGGATT**YK**TCCTTCTCCAG | | | | | |
| Ruff50 | AF473576 | Standard | F: GCTGTCAATATGCCATTGGTAACAT | 54-66 | 2.0 | 0 | 1 | 0 |
| | | Standard | R: TTGCAACAGAAACCCATATAAGCAT | | | | | |
| 9E6* | AM600674 | Standard | F: CTCCTGGGTGATTGGT | 54-56 | 2.0 | 0 | 0 | 0 |
| | | Standard | R: GATCCGTCTGCTAGGG | | | | | |
| Apy02* | AJ391209 | Standard | F: GGAAATTCTTTCCTGAAGTCTCC | 54 | 2.0 | 0 | 0 | 1 |
| | | Standard | R: AAAATCTGAGACTATCACCTTTATCAT | | | | | |
| BmaGGAT313* | DQ173178 | Standard | F: ACCTCTAAAGGTCCCTTCCAACC | 54-66 | 2.0 | 0 | 0 | 0 |
| | | Standard | R: GTATGGCTCTTCAGGTATTCCCAGT | | | | | |
| BmaCA382* | DQ173192 | Standard | F: GGCCTCTTTCTTCATGGGTCTTAT | 54-56 | 2.0 | 1 | 1 | 0 |
| | | Standard | R: ACAGCTCTTCCCAATGACTGCT | | | | | |
| Calex-24* | AM072476 | Standard | F: GATCTTGGCTGTGCACAGG | 62-66 | 2.0 | 1 | 0 | 0 |
| | | Standard | R: GTTTCTTGCCCTTTGAAGAGAGGAGGA | | | | | |
| Calex-37* | AM072492-3 | Standard | F: CCATTTGCAGCTGTAACATAAAGGTCT | 54-55 | 2.0 | 1 | 0 | 0 |
| | | Standard | R: GTTTCTTGATCCCCTCCTGCTGGTCCT | | | | | |
| K31* | AY083598 | Standard | F: GTATTCTCCTCCCGTCGGTGTT | 54-59 | 2.0 | 0 | 0 | 0 |
| | | Standard | R: GCCTGTCCTTGTCCTGCTCTT | | | | | |
| K67* | AY083601 | Standard | F: AAAACGGGGTTCTCCTCCCTAC | 54-56 | 2.0 | 0 | 0 | 0 |
| | | Standard | R: ACACCGGGGAGGAACAATG | | | | | |
| LarsNX01* | DQ251028 | Standard | F: GCTGATAGTCCCTTTAGTAGACTTTA | 54-56 | 2.0 | 0 | 0 | 0 |
| | | Standard | R: AATGGCAGGAGAACTTCC | | | | | |
| Sdaat46* | AY597043 | Standard | F: TTGTTGACTCGTTTGAGTTCCTTTC | 54-56 | 2.0 | 0 | 1 | 1 |
| | | Standard | R: TGTTTGTGGGTAGACAGCTTATGGT | | | | | |

*anonymous clones. These clones could not be assigned to a homolous sequence in the chicken genome

†degenerate bases in bold letters (K=G/T, M=A/C, R=A/G, S=C/G, W=A/T, Y=C/T)

‡temperature range in which amplification success was maximal for Kentish plover, whiskered auklet and ruff

Amplification: 0 amplification failed or no specific bands; 1 amplification succesful, clear band visible on agarose gel

[1]microsatellite homologue to locus LarsZAP19 (Gregory and Quinn 2006)

[2]microsatellite homologue to locus K32 (Tirard et al. 2002)

[3]microsatellite homologue to locus LarsNX24 (Gregory and Quinn 2006)

[4]mirosatellite homologue to locus LarsZAP11 (Gregory and Quinn 2006)

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Chapter IV

# Kentish versus snowy plover: Phenotypic and genetic analyses of *Charadrius alexandrinus* reveal divergence of Eurasian and American subspecies

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**Contributions:**

**C. Küpper:** set up of microsatellite analysis, mitochondrial analysis, phylogenetic and statistical analyses, manuscript preparation
**J. Augustin:** microsatellite genotyping, manuscript improvement
**A. Kosztolányi:** sample acquisition, manuscript improvement
**T. Burke:** manuscript improvement
**J. Figuerola:** manuscript improvement
**T. Székely:** sample acquisition, manuscript improvement

## Abstract

Many shorebird species have widespread geographic distributions comprising several continents. Because shorebirds also have excellent dispersal capabilities and can migrate large distances, it is often unclear whether there are reproductive barriers between subspecies and populations from different continents. Kentish / snowy plovers *Charadrius alexandrinus* are cosmopolitan shorebirds. There has been a longstanding debate about whether the American and Eurasian subspecies (snowy and Kentish plover, respectively) constitute a single species. We examined the divergence between American and Eurasian populations to reassess the current taxonomy by comparing phenotypic and genetic characters of the American subspecies *nivosus* and the Eurasian subspecies *alexandrinus* from seven populations. We found several diagnostic morphological characteristics that show consistent differences between snowy and Kentish plovers. In particular, snowy plovers had significantly shorter tarsi and wings than Kentish plovers. Kentish and snowy plover also differed in downy chick plumage and adult courtship calls. Genetic analyses revealed that American and Eurasian populations have strongly diverged, with the Kentish plover being more closely related to the white-fronted plover *C. marginatus* than to the snowy plover. These results were consistent across all assessed nuclear markers (26 microsatellites and a partial *CHD* sequence) and three mitochondrial loci. Within the subspecies populations sampled across large geographic distances did not show signs of differentiation, with $F_{st}$ and $\Phi_{st}$ values between populations not differing from zero. The combined results suggest that the taxonomic status of *C. alexandrinus* needs to be revised and we propose that Kentish plover and snowy plover should be recognised as separate species.

## Introduction

Cosmopolitan species have widespread geographic distributions and can be found on most continents or in most oceans. Many cosmopolitan species are found among parasites, invertebrates, marine vertebrates, and organisms with small body sizes (Klautau *et al.* 1999, Fenchel & Finlay 2004, Bleidorn *et al.* 2006), but among terrestrial vertebrates cosmopolitans are rare, with the exception of those that have been introduced by humans.

Until recently, species have often been defined according to highly consistent morphologically characters that are shared by a group of individuals. However, when molecular characters were included into their taxonomic evaluation, many of these morphospecies have been found to consist of several cryptic species (e.g. Klautau *et al.* 1999, Bleidorn *et al.* 2006, Bickford *et al.* 2007). Cryptic species have been found across many different metazoan taxa. For example, in birds 94 cryptic species complexes were identified between 1978 and 2006 (Pfenniger & Schwenk 2007).

Shorebirds, gulls, terns and auks (*Charadriiformes*) harbour many species with outstanding migration abilities and widespread geographical distributions (del Hoyo *et al.* 1996). Among shorebirds, there are only two species with a cosmopolitan distribution that breed in temperate and subtropical climate zones: black-winged stilts (*Himantopus himantopus*) and Kentish plovers (*Charadrius alexandrinus)* (Hayman *et al.* 1986, del Hoyo *et al.* 1996). Due to their widespread distribution and morphological differentiation into many subspecies, the taxonomy and phylogeography of both species deserve special attention.

Our understanding of the phylogeny and taxonomy of *Charadriiformes* has advanced rapidly since the introduction of new molecular and computational intensive methods (e.g. Paton *et al.* 2003, Ericson *et al.* 2003, Thomas *et al.* 2004a, b, Baker *et al.* 2007). However, there are still many unresolved questions regarding the exact phylogenetic relationships within and between most shorebird species. These unresolved relationships hamper our understanding of central problems in evolutionary biology that rely on a correct phylogeny, such as the evolution of mating and parental care systems (Székely & Reynolds 1995, Thomas & Székely 2005, Thomas *et al.* 2006).

The Kentish plover, first described by Linnaeus in 1758, breeds in temperate and subtropical regions of North and South America, Africa, Europe and Asia. Migratory Kentish plovers also reach Australia during the nonbreeding season (BirdLife International 2007). Recently, Kentish plovers have attracted considerable attention in evolutionary and conservation biology because of their flexible breeding system that varies across and within populations (Lessells 1984, Warriner *et al.* 1986, Székely & Williams 1995, Amat *et al.* 1999, Kosztolányi *et al.* 2006, Székely *et al.* 2006), and because many populations are fragmented and declining (Stroud *et al.* 1994, Page *et al.* 1995).

The taxonomic classification of Kentish plover populations is still debated. The Kentish plover is considered by some authors to comprise a superspecies with white-fronted plover *C. marginatus* and red-capped plover *C. ruficapillus* (Hayman 1986, Sibley & Monroe 1990). Some authors also include the Javan plover *C. javanicus* into this superspecies complex (Rittinghaus 1961, del Hoyo *et al.* 1996). Interestingly, the other members of the superspecies are all currently considered to be monogamous, whereas Kentish plovers are usually polyandrous. Six to ten Kentish plover subspecies are recognised, with most authors generally acknowledging six subspecies: *C. a. nivosus, C. a. tenuirostris* and *C. a. occidentalis* inhabit North and South America and are commonly called snowy plovers. The other three acknowledged subspecies *C. a. alexandrinus, C. a. dealbatus* and *C. a. seebohmi* breed in Eurasia (del Hoyo *et al.* 1996, Cramp & Simmons 1983, Rittinghaus 1961). The subspecies classification of snowy plovers was recently supported by mitochondrial and microsatellite analyses (Funk *et al.* 2007).

Whether American and Eurasian subspecies belong to the same species has been subject of an ongoing debate. Historically, the snowy plover was originally considered to be a separate species (described as *Aegialitis nivosa* by Cassin 1858, cited in Oberholser 1922). Later, snowy plovers were 'downgraded' into three subspecies within the Kentish plover, because the differences in adult plumage were not consistent (Oberholser 1922). The latter proposition was accepted by Monroe and Sibley (1993), although Sibley and Monroe (1990) commented that the snowy plover may deserve full species status.

The aim of this study was to investigate population differentiation using both phenotypic and genetic characters of both American and Eurasian *C. alexandrinus* populations to evaluate the current taxonomic status. First, we compared phenotypic characters such as biometric

measurements, chick plumage and courtship calls between Kentish and snowy plover. Second, we examined molecular characters of snowy and Kentish plover populations and compared them to molecular characters of a white-fronted plover population. The closely related white-fronted plover served to compare the magnitude of genetic differences between snowy and Kentish plover with those between the two recognised species of Kentish and white-fronted plover.

## Materials and methods

### *Biometry*

We collected data on three biometric characters (tarsus length, wing length and body mass) of adult snowy and Kentish plovers from five populations during the breeding season (Fig. 1): snowy plovers were measured at Ceuta (Mexico, N 23°57, W 106°59, collected in 2006–07), whereas Kentish plovers were measured at Miklapuszta (Hungary, N 46°40, E 19°10, collected in 1992-94), Tuzla (Turkey, N 36°42, E 35°03, collected in 1997–99), Doñana (Spain, N 36°56, W 6°21, collected in 2004) and Al Wathba (United Arab Emirates [UAE], N 24°16, E 54°36, collected in 2005–06).

We measured tarsus and wing length to the nearest 0.1 mm. If measurements of both limbs were available, we used their mean in the analysis. Body mass was measured to the nearest 0.1 g using Pesola spring balances.

We randomly selected 40 breeding males and 40 breeding females from each population and used two-way analyses of variance (ANOVA) with population and sex as factors for adults to examine biometric differences between plover populations. Non-significant interactions were removed from the final model. To compare biometrics specifically between snowy and Kentish plovers we used a contrast analysis in which we *a priori* contrasted measurements from snowy plovers (Ceuta) with measurements from Kentish plovers (Al Wathba, Tuzla, Doñana, Miklapuszta). If the population means tested by the ANOVAs differed significantly, we further examined the differences by post-hoc Tukey tests. Breeding latitude may strongly influence the body mass of individuals (Blackburn *et al.* 1999) and the biometric measurements of snowy plover were taken from a population breeding at low latitudes. Therefore, we used a Generalized Linear Model (GLM) to examine whether latitude influenced body mass using the body mass data from Kentish plovers for which body mass from several locations was available.

We also compared the tarsus lengths and body masses of 16 randomly selected chicks per population, that had been measured on the day of hatching, using one-way ANOVAs with the contrast methods outlined above. For statistical analyses we used R version 2.4.1 (R Development Core Team 2006).

***Calls***

We obtained Kentish and snowy plover courtship calls of males from bird-song collectors. Snowy plover calls recorded in North Dakota were provided by Lang Elliot. Kentish plover calls were obtained from two sources: i) the British Library Sound Archive, which supplied records of advertising males from breeding populations in Tunisia, Greece and the Netherlands, and ii) Jean Roché, who provided male courtship calls from Kentish plovers breeding in France. Sonograms were prepared using SAS Lab Light software (Avisoft).

***DNA collection and preparation***

We obtained DNA samples from seven populations including four Eurasian (Fig. 1, Doñana, Tuzla, Al Wathba and Kujalnik [Ukraine, N 46°45, E 30°36), two American populations (Fig. 1, Ceuta and Great Salt Lake [USA at N 41°00, W 112°00]) and one population of breeding white-fronted plovers from the west coast of Madagascar (Fig. 1, S 15°50, E 45°57).

To obtain DNA samples, adult plovers were trapped on the nest during incubation using funnel traps (Székely *et al.* 2008). We obtained a small blood sample (25–50 µl) from the brachial vein. Blood was stored either in Queen's lysis buffer (Seutin *et al.* 1991) or absolute ethanol until extraction. All samples were collected between 1997 and 2006 (see also Blomqvist *et al.* 2002, Küpper *et al.* 2004).

DNA was extracted using an ammonium acetate (Nicholls *et al.* 2000), a salt acetate (Bruford *et al.* 1998) or an adapted phenol–chloroform method (Krokene *et al.* 1996). All DNA samples were extracted by CK and JA except for samples from Salt Lake, which were kindly provided by Tom Mullins and Susan Haig (USGS Forest and Rangeland Ecosystem Science Centre, Corvallis). Extracted DNA was visualised on a 0.8% agarose gel stained with SYBRsafe (Invitrogen) to assess DNA quality. DNA quantity was estimated by measuring the optical density of samples at 260 nm using a Fluostar Optima fluorimeter (BMG laboratories).

***Mitochondrial DNA***

We amplified three mitochondrial markers: an approximately 400-bp long NADH dehydrogenase subunit 3 fragment (using the L10755 and HI1151 primers, Chesser 1999), an approximately 1.2-kbp sequence including partial fragments of the ATPase subunit 8 and subunit 6 genes (using the CO2GQL and CO3HMH primers, Eberhard & Bermingham 2004) and an approximately 700-bp section of the control region (using primers SNPL90, Funk *et al.* 2007 and TS778H, Wenink *et al.* 1994).

We amplified fragments using 20-µl Polymerase Chain Reactions (PCRs) that contained 20 ng of DNA and 0.5 units of Taq DNA polymerase (Bioline) in the manufacturer's buffer with a concentration of 1.0 µM of each primer, 2.0 µM MgCl2 and 0.20 mM of each dNTP. PCRs were carried out on a thermal cycler (MJ Research model PTC DNA engine) using the following program: one cycle of 3 min at 94°C followed by 35 cycles of 94°C for 30 s, annealing temperature of 55°C for 30 s, 72°C for 30 s, and a final extension cycle of 10 min at 72°C. To check for amplification success, we visualized 5 µl of each PCR product on a 2% agarose gel stained with SYBRsafe (Invitrogen).

Successful PCR products were cleaned up and precipitated with ethanol and sequenced using Big Dye Terminator Cycle chemistry on ABI 3730 capillary DNA automated sequencers. Samples from Doñana were sequenced at the Natural Environmental Research Council (NERC) Molecular Genetics Facility (MGF) at the University of Sheffield, whereas all other samples were sequenced at the NERC MGF at the University of Edinburgh. The location of the sequencing did not affect the results (see Results), since two haplotypes (one haplotype shared by individuals ALW23, ALW25, DON27 and DON34, and another haplotype shared by individuals DON33 and TUZ38) were found among individuals sequenced in both Sheffield and Edinburgh. Sequences were manually edited using CodonCode Aligner version 2.0.0 beta 7. Only partial sequences with both forward and reverse strands available were used in subsequent analyses. In total, a 384-bp partial sequence of the *ND3* gene, a 399-bp sequence of the *ATPase* subunit 6 and partial subunit 8 genes, and a 498-bp partial sequence of the control region (1281 bp in total) for each of 53 individuals across the seven populations were available for the subsequent analysis (for frequency distribution see Table 3). Sequences were aligned using the CLUSTALW algorithm implemented in CodonCode Aligner 2.0.0 beta 7.

The use of avian blood as a DNA source might lead to amplification of nuclear pseudogenes. We confirmed amplification of the targeted mitochondrial regions in two ways. First, we translated the coding mitochondrial sequences into peptide sequences using the EBI–Transeq tool available from http://www.ebi.ac.uk/emboss/transeq/. Peptide sequences of the coding sequences of *ND3* and *ATPase 6/8* did not show any unexpected stop codons that would have been characteristic for pseudogenes. Second, we conducted searches for highly similar sequences using the nucleotide BLAST program available from the NCBI webpage (http://www.ncbi.nlm.nih.gov/blast/) against the 'nucleotide collection' database using the sequences we obtained from a male snowy plover (CEU6, AM941499, AM941552, AM941605). We anticipated that nuclear pseudogenes should show little similarity to other *Charadriiformes* mitochondrial sequences and hence pseudogene amplification products could be recognised by low identity values in the BLAST results. The results showed high similarity between our sequenced fragments and records of mitochondrial markers from other *Charadriiformes*. The haplotype of CEU6 showed an identity of 92% to the *ATPase* subunit 8 and subunit 6 sequences of several mountain plover records (*C. montanus,* e.g. AY794551), 86% identity to ND3 gene of a stone curlew record (*Burhinus oedicnemus,* AF076345) and 99–100% identity with the mtDNA control region of other snowy plover records (e.g. EF215690). As all the comparisons were highly similar (see Table 2) we are confident that we amplified the mitochondrial targets.

Before the phylogenetic and population genetic analysis, we tested sequence homogeneity within the concatenated haplotype sequences using the partition homogeneity test with 100 replications in PAUP*4.0b10 (Swofford 2000). The test results ($P$ = 0.94) did not indicate any significant conflicts. Therefore we used the concatenated sequence consisting of a total length of 1281 bp for parsimony and population genetic analyses.

For the parsimony analyses a heuristic search with 300 random addition-sequence replicates and TBR branch swapping was carried out in PAUP*. Nodal support was assessed through nonparametric bootstrap analysis using 1,000 bootstrap replicates, each with 100 random addition-sequence replicates and tree bisection and reconnection (TBR). For the Bayesian analysis, the most appropriate model of sequence evolution was selected using Akaike's information criterion (Akaike 1974) in MRMODELTEST 2.2 (Nylander 2004). The Bayesian analysis was conducted using

MRBAYES 3.1 with data partitioned according to the different mtDNA markers (Huelsenbeck & Ronquist 2001). The default settings (two Markov chains at four different temperatures) were used. Markov chains were sampled every 100 generations and run for five million generations. Trees were drawn using the program TREEVIEW version 1.6.6 (Page 1996).

Population genetic analyses for mitochondrial markers were performed using ARLEQUIN version 3.1 (Excoffier *et al.* 2005). Genetic variation within populations was estimated using several diversity statistics, including haplotype diversity (*h*), number of polymorphic sites (*s*), and nucleotide diversity ($\pi$). Corrected mean percentage sequence divergences between populations and the three major groups (Kentish plover, snowy plover and white-fronted plover) were calculated. Genetic divergence among populations was estimated by $\Phi$-statistics and analysis of molecular variance (AMOVA; Excoffier *et al.* 1992), which takes into account the number of mutations between haplotypes. A permutation test with 1,000 randomly generated $\Phi_{st}$ values was used to test the probability of observed $\Phi_{st}$ values arising by chance. Pairwise $\Phi_{st}$ values were calculated between all seven populations and between the putative three major groups. Significance levels were adjusted using the sequential Bonferroni method (Rice 1989). AMOVAs were carried out to compare the variance components explained by the major groups, populations and individuals. One thousand random permutations were also used to test for the significance of variance components.

Mitochondrial sequences were deposited in the European Molecular Biology Laboratory database under accession numbers AM941499 – AM941657.

***Nuclear markers***

Fragment length differences in nuclear markers were examined in 166 individuals from the seven plover populations using a sex-specific marker located in the chromohelicase DNA binding protein (*CHD*) gene and 26 autosomal microsatellite markers.

In many birds, the sexes can be distinguished according to the specific product sizes of an amplified *CHD* PCR product (Griffiths *et al.* 1998). Product sizes of *CHD* fragments are not only sex chromosome specific, but often also species specific (see D.A. Dawson's bird sex-marker web page: http://www.shef.ac.uk/misc/groups/molecol/BirdsexingSMGF.xls). To examine whether Kentish, snowy and white-fronted plover differ in the W or Z-*CHD* product size we amplified a

partial sequence of the *CHD* gene using NED-labelled sexing primers (*P2 / P8* primers, Griffiths *et al.* 1998). The primers were part of the multiplex reaction (MR 1, conditions see below).

Population and group differentiation were further tested using 26 microsatellite markers. The markers were chosen for polymorphism and their ability to be arranged in multiplex PCRs. All 21 *Calex* primer pairs were designed from microsatellite loci isolated in Kentish plovers (*Calex* primers, sequences in Küpper *et al.* 2007). Additionally, four primer sets developed for snowy plovers (*C201, C203–C205,* Funk *et al.* 2007) and one primer set developed for barn swallows *Hirundo rustica, (Hru2,* Primmer *et al.* 1995) were used.

Each sample was run in four multiplex PCRs (MR 1–4) containing different combinations of fluorescently labelled primers (fluorescent labels are given in Table 5). MR 1 contained the primers *Calex-02, -04, -05, -08, -18, -19, -23, -24, -39, -43, -45* and the *P2 / P8* primer set. MR 2 contained primers *Calex-01, -11, -12, -14, -22, -28* and *Calex-37.* MR3 contained primers *Calex-10, Calex-32, -35, C201* and *C203*. MR4 contained primers *C204, C205* and *Hru2*.

Ten-µl MRs contained 8 µl mastermix solution (Qiagen), approximately 2 µM of the primer mix and 10 ng DNA. Relative primer concentrations were optimised to obtain similar peak sizes across different primer sets in the fragment analysis. MRs were performed in a thermal cycler (MJ Research model PTC DNA engine) according to the multiplex kit manufacturer's default protocol: the program started with a 15-min activation cycle at 95°C followed by 35 cycles of 94°C for 30 s, annealing temperature (MR 1: 57°C, MR 2: 62°C, MR 3 & 4: 60°C) for 90 s, and 90 s at 72°C. The program finished with a 10-min extension cycle at 72°C. A fraction of the MR products was loaded onto the ABI 3730 and allele sizes were assigned using GENEMAPPER 3.7 software (Applied Biosystems).

Hardy-Weinberg equilibrium and linkage disequilibrium between markers were tested in GENEPOP version 3.3 (Raymond & Rousset 1995) using the Kentish plover samples from Tuzla (*n* = 30 individuals) for which most markers had been developed (Küpper *et al.* 2007). The sequential Bonferroni method was applied to correct for multiple testing.

Heterozygosity corrected for sample size (Nei 1978) was calculated in SPAGeDi 1.2 (Hardy & Vekemans 2002). Heterozygosities were compared using a nonparametric Kruskal-Wallis test followed by a post-hoc Wilcoxon rank sum tests with the sequential Bonferroni correction to identify population pairs that differed significantly.

Genetic divergence was assessed by first calculating pairwise $F_{st}$ values between populations and major groups and, second, by an AMOVA analysis using ARLEQUIN 3.1 (Excoffier *et al.* 2005). As with mtDNA, the significance of $F_{st}$ values was tested using a permutation test with 1000 random permutations.

Two Bayesian clustering approaches were used to examine population differentiation. First, we tested differentiation with the program STRUCTURE version 2.1 (Pritchard *et al.* 2000) to estimate the number of clusters $K$ and to assign individuals to one or more of these clusters. We used the admixture model, which assigns a proportion of each individual's genome to each population assuming gene flow among populations. The likelihood for each number of clusters ranging from $K = 1$ (complete panmixia) to $K = 7$ (maximum divergence) was calculated assuming correlated allele frequencies. Five independent simulations with a burn-in length of 50,000 and a run length of 100,000 generations were conducted for each $K$.

Second, we used the program BAPS version 5 (Corander *et al.* 2003) to run five iterations of a population mixture analysis. The program calculates first the likelihood for the number of clusters ($K = 1$ to 7). This was followed by a population admixture analysis to assign genotypes to the different clusters with a probability $\geq 0.95$.

## Results

### *Biometry*

The morphometric body characteristics of plovers differed between populations after controlling for sex (Table 1, Fig. 2). The contrast analysis revealed that these differences were largely because snowy plovers were smaller than Kentish plovers (Table 1, Fig. 2, contrast analysis: tarsus length: $t_{4, 395} = 26.68$, $P < 0.001$; wing length: $t_{4, 395} = 7.68$, $P < 0.001$; body mass: $t_{4, 395} = 9.18$, $P < 0.001$). There was no significant difference in tarsus length between Kentish plover populations (in all tests $P > 0.25$). Wing length differed between Kentish plover populations, with plovers from either Tuzla or Al Wathba having significantly shorter wings than plovers from either Doñana or

Miklapuszta (post-hoc Tukey test: each comparison $P < 0.01$). However, Kentish plovers from three of the four sampled populations had significantly longer wings than snowy plovers from Ceuta (Table 1, post-hoc Tukey tests comparisons with Ceuta for Al Wathba, Doñana, Miklapuszta, all tests with $P < 0.001$), and there was a strong trend for Kentish plovers from the fourth population (Tuzla) to have longer wings than snowy plovers from Ceuta (Table 1, post-hoc Tukey test: $P = 0.06$). The body mass of Kentish plovers was strongly associated with latitude: Kentish plovers breeding at higher latitudes were heavier than Kentish plovers breeding at lower latitudes (GLM: $t_{1, 318} = 18.55$, $P < 0.001$).

The body sizes of freshly-hatched snowy and Kentish plover chicks were also different. Snowy plover chicks were smaller than Kentish plover chicks. Snowy plover chicks had shorter tarsi and lighter body masses than Kentish plover chicks from all sampled locations (Table 1, Fig. 2, contrast analysis: tarsus: $t_{75} = 5.96$, $P < 0.001$; body mass: $t_{75} = 3.40$, $P = 0.001$). Latitude and body mass were negatively associated in Kentish plover chicks, with chicks hatching at lower latitudes being heavier than chick hatching at higher latitudes (GLM: $t_{1, 62} = -2.44$, $P = 0.02$). The results of post-hoc Tukey tests showed no significant differences in body mass for Kentish plover hatchlings (in all tests $P > 0.09$), but revealed further significant differences in hatchling tarsus length between Kentish plover populations. Chicks from Al Wathba had shorter tarsi than chicks from Miklapuszta ($\Delta$ mean = 1.32 mm, $P < 0.001$) and chicks from Tuzla ($\Delta$ mean = 0.93 mm, $P = 0.03$). However, there was a trend for even the smallest Kentish plover chicks that originated from the Al Wathba population to have longer tarsi than the snowy plover chicks from Ceuta (post-hoc Tukey test: $\Delta$ mean = 0.74 mm, $P = 0.12$).

***Plumage***

Chicks of snowy and Kentish plovers differed in their downy plumage. Kentish plover chicks showed a characteristic dark central stripe on the back, whilst snowy plover chicks lacked this central stripe (Fig. 3).

***Call***

The male courtship calls were similar between Kentish plover recordings. However, courtship calls of snowy and Kentish plovers showed remarkable differences (Fig. 4). Snowy plover males

advertise with a long 'purr'-call, whilst a sharp metallic motif is repeated several times by advertising Kentish plover males.

***Mitochondrial markers***

Among the 1281 characters of the concatenated mtDNA sequence, 123 bp were informative for the parsimony analysis. The mean base frequencies of the total sequence were Adenosine: 28.0%, Cytosine: 25.6%, Guanine: 18.4% and Thymine 28.0%.

Forty distinct haplotypes were found among the 53 individuals for which the full sequence was available. Six haplotypes belonged to 13 sequenced snowy plovers, three haplotypes were present in four white-fronted plovers and 31 haplotypes were found among 36 Kentish plovers. Haplotypes were shared between individuals of different populations within, but not between, the three major groups.

Parsimony analysis generated 127 maximum parsimonious trees with 158 steps. An unrooted network generated from the strict consensus tree showed three main branches, consistent with the geographical distributions of snowy, white-fronted and Kentish plover (Fig. 5). No substructures were found within any of these three groups.

The most appropriate model for sequence evolution was the *TrN* + *Γ*+ *I* model (Tamura & Nei 1993) and this was consequently chosen for the Bayesian mtDNA analysis. In this analysis, likelihood values converged after about 50,000 generations. Before constructing the tree, we removed a conservative burn-in period of 1,250,000 generations (25%) according to the MRBAYES manual. The consensus tree showed the same main branches as the consensus parsimony tree: all snowy plover mitochondrial sequences segregated on a separate branch from the combined Kentish and white-fronted plover branch (clade credibility equals 1). Clade-credibility values for separating white-fronted plover and Kentish plover were higher than 0.95; however, most intrataxon groupings were not well supported. Only three groups within the group of Kentish plovers showed credibility values higher than 0.95, but these groupings were not associated with the geographical origins of individuals.

The net sequence differences of mtDNA markers were significantly different between snowy, white-fronted and Kentish plovers (Table 2). Kentish and snowy plovers differed more than Kentish

and white-fronted plovers, whereas the largest difference was found between snowy and white-fronted plovers (Table 2). Interpopulation comparisons within the *alexandrinus* or *nivosus* subspecies did not reveal any significant differences between any pairs of populations (Table 2).

Haplotype diversity was highest in Kentish plover populations, with all four populations reaching diversity levels above 0.95, whereas white-fronted plover and snowy plover populations had lower haplotype diversity, with the strongly reduced diversity in haplotypes from snowy plovers sampled in the Salt Lake region in Utah, where four out of five individuals shared the same haplotype (Table 3).

$\Phi_{st}$ values among the three groups were highly significant (Table 4). The results were consistent with highly significant pairwise $\Phi_{st}$ values for comparisons between single populations of the three groups ($P < 0.001$). Within groups, $\Phi_{st}$ values were low, ranging from 0.01 (Al Wathba vs Tuzla) to 0.17 (Al Wathba vs Kujalnik), and none of the pairwise interpopulation comparisons was significant.

The AMOVA revealed that most of the mtDNA variation was attributable to the differences between the three groups ($df = 2$, explained variation = 94.5%, $P < 0.001$). Very little variation was explained by population ($df = 4$, explained variation = 0.6%, $P = 0.02$) and little variation was harboured within populations ($df = 46$, explained variation = 4.9%, $P = 0.01$).

***Nuclear markers***

The size of *CHD-W* fragments among all plover females was 381 bp ($n = 91$). However, the sizes of *CHD-Z* fragments differed. The typical size for the amplified *CHD-Z* fragment was 373 bp in Kentish ($n = 112$ individuals) and white-fronted plovers ($n = 19$ individuals). Two male Kentish plovers, one from Tuzla and one from Al Wathba, were heterozygous with an additional Z allele of estimated size 371 bp in addition to their 373-bp allele. The *CHD-Z* fragment in snowy plovers was consistently shorter, with a length of 365 bp ($n = 35$ individuals), and all males were homozygous.

The microsatellite allele size ranges differed between populations at three loci (Table 5). One marker, *Calex-28* did not amplify in snowy plovers, although it amplified in 110 of 112 Kentish and

15 of 19 white-fronted plovers. Allele sizes did not overlap between snowy plover and Kentish / white-fronted plover at loci *Calex-10* and *Calex-39* (Table 5).

One marker (*Calex-10*) had significant homozygote excess in Kentish plovers breeding in Tuzla. Significant linkage disequilibria were detected between three pairs of microsatellite loci: *Calex-02* and *C201*, *Calex-23* and *C203*, and *C204* and *C205*. Therefore, we ran two independent batches for all analyses of microsatellite data: the first including all markers and the second excluding *Calex-10, C201, C203* and *C205*. Both analyses gave similar results and we present only the results including all 26 microsatellite markers.

Genetic variation (microsatellite heterozygosity corrected for sample size according to Nei (1978)) was different across plover populations (Table 5, Kruskal Wallis test: $\chi^2 = 103.80$, $df = 6$, $P < 0.001$). Heterozygosities did not differ between snowy and white-fronted plover populations (Wilcoxon rank sum tests: in all possible comparisons, $P > 0.46$). However, the four Kentish plover populations harboured substantially more genetic variation than the two snowy plover populations (Wilcoxon rank sum tests: in all pairwise comparisons $P < 0.001$). All loci were polymorphic in Kentish plover populations, but not in white-fronted and snowy plover populations (Table 5).

$F_{st}$ values for microsatellites revealed strong differentiation between Kentish, snowy and white-fronted plovers (Table 4). Consistent with the mitochondrial results, within groups the pairwise $F_{st}$ values were not significantly different from zero (Table 4). The AMOVA showed that a large proportion of the variation in allele frequencies was explained by the three putative main groups ($df = 2$, explained variation = 33.6%, $P < 0.001$). As with mitochondrial DNA, little variation was explained by the actual population membership (explained variation = 0.4%, $df = 4$, $P = 0.014$). However, in contrast to the mitochondrial DNA, the largest part of the variation was harboured within populations (explained variation = 66.0%, $df = 325$, $P = 0.007$).

Log-likelihood values for the Bayesian analyses in STRUCTURE reached a plateau at $K = 3$, with one cluster for each major group. Individual genomes were largely assigned into clusters according to their group origin. In four out of five iterations all genomes were assigned to their respective cluster, with at least 0.98 proportional memberships. In the fifth run all snowy and white-fronted plovers were assigned to a single cluster, with average proportional memberships of 0.99 and 0.88,

respectively, whereas the genomes of Kentish plovers were assigned to two other clusters. In the latter run average proportional memberships for Kentish plover genomes were poor: in all populations smaller than 0.62. Interestingly, when we set $K = 2$, Kentish plover and snowy plovers were always assigned to different clusters, whilst white-fronted plovers were assigned either to the Kentish (n = 1 replicates, average proportional membership = 0.99) or snowy plover cluster (n = 4 replicates, average proportional membership = 0.90).

The results obtained with BAPS were consistent with the results from STRUCTURE. The marginal log-likelihood for three clusters was –14,367 (probability = 1.00). The assignment probability for each individual genome in the mixture analysis was 1.00, based on 50 simulations from posterior allele frequencies.

## Discussion

Our study produced four major results regarding the taxonomy and phylogeography of Kentish, snowy and white-fronted plovers. First, in contrast to the current classification of *Charadrius alexandrinus* as a single cosmopolitan species, we found consistent phenotypic and genetic differences between Kentish and snowy plovers. Second, the magnitude of genetic differences between Kentish, snowy and white-fronted plover measured by both mitochondrial and nuclear markers suggests that the split between Kentish and snowy plovers occurred earlier than the split between Kentish and white-fronted plovers. Third, Kentish plovers (*C .a. alexandrinus*) showed higher genetic diversity in mitochondrial haplotypes and microsatellite markers than snowy or white-fronted plovers. Fourth, despite large distances between sample locations of several thousand kilometres in both snowy (subspecies *nivosus*) and Kentish plovers (subspecies *alexandrinus*), we detected no population differentiation within each subspecies.

### *1. Phenotypic differences between Kentish and snowy plovers*

The biometric measurements of tarsus and wing length showed that Kentish plovers are consistently larger than snowy plovers. On average, Kentish plovers were also significantly heavier than snowy plovers. However, males and females of Kentish plovers breeding in Al Wathba were lighter than snowy plovers from Ceuta breeding at a very similar latitude. Adult body mass may not therefore be a defining difference between the two groups. The high body mass of Kentish plovers breeding in Germany corroborates a strong latitudinal trend consistent with the 'neo-Bergmann rule' (Blackburn *et al.* 1999). Plovers breeding at higher latitudes are heavier than plovers breeding at

lower latitudes, possibly because of more metabolically favourable mass / surface area ratios (Bergmann 1847, Rensch 1938, James 1970, Blackburn *et al.* 1999). Surface temperatures in Al Wathba often exceed 60°C at midday on the breeding grounds (Alrashidi *et al.*, submitted) and adult plovers appear to be heat stressed and may lose substantial water during the day or may strategically lower their body mass to cope with the heat. Interestingly, we found the opposite trend in chicks: body mass and latitude on the breeding grounds were negatively associated in Kentish plover hatchlings, which may suggest differential investment into the eggs by females.

Chick plumage differed between Kentish and snowy plovers. Kentish plover chicks have a dark stripe in the middle of the back that is missing in snowy plovers. Patterns of downy plumage showed consistent differences between shorebird species and have been used to construct a shorebird phylogeny (Jehl 1968) and can therefore serve as suitable character to separate snowy and Kentish plovers.

Adult plumage in snowy and Kentish plovers shows more variation within and across subspecies, which was the main reason why both groups were merged into a single species (Oberholser 1922). Snowy plovers are generally paler than Kentish plovers. Snowy plover males have brown head caps whilst Kentish plover males usually have a buff-orange head colouration (Hayman *et al.* 1986). However, the males of one Kentish plover subspecies (*C. a. seebohmii* Whistler 1937, cited in Rittinghaus 1961) also lack the reddish crown, so this may not serve as a suitable defining feature. We noticed during our field studies in Mexico and Eurasia that sexual dimorphism is reduced in snowy plovers. In the beginning of the breeding season female snowy plovers exhibit black head and breast badges that make them almost indistinguishable from males, whilst Kentish plover are clearly sexually dimorphic in the beginning of the breeding season.

In contrast to their adult plumage, male Kentish and snowy plovers could be clearly distinguished by their calls.

***2. Genetic differences between Kentish, snowy and white-fronted plover***

The phenotypic differences were matched by a number of genetic differences between both groups. Examination of mitochondrial and nuclear markers (*CHD* and microsatellite loci) showed that Kentish and snowy plovers have consistent genetic differences. This indicates that the current oceanic barriers prevent detectable gene-flow between the Eurasian and American populations. The

magnitude of the genetic differences suggests that gene flow has been absent for a considerable time.

We found good support from all genetic markers for the hypothesis that snowy plovers diverged from Kentish plovers before the divergence of Kentish and white-fronted plovers. Differences between mitochondrial DNA sequences and *CHD-Z* genotypes were larger between Kentish and snowy plovers than between Kentish and white-fronted plovers. In trees obtained from mitochondrial phylogenetic analyses by either parsimony or Bayesian methods, snowy plovers diverged first from the Kentish / white-fronted plover clade. This initial split was also supported by the microsatellite analysis, because microsatellite allele ranges completely overlapped between the white-fronted plover and Kentish plover, but were distinct between snowy and Kentish plovers at two loci. One further marker, *Calex-28*, consistently failed to amplify in snowy plovers, whilst it did amplify in Kentish and white-fronted plovers. Both $F_{st}$ and $\Phi_{st}$ values were larger between snowy and either white-fronted or Kentish plover populations than between Kentish and white-fronted plover populations, although the differences were not large. Finally, when we forced the Bayesian analysis in STRUCTURE to assume there were only two clusters, it consistently placed Kentish and snowy plovers in separate clusters.

***3. Genetic diversity***

Populations showed consistent differences in genetic diversity according to their origin. All four Kentish plover populations harboured high genetic diversity as measured by microsatellite and mitochondrial DNA markers, whilst the diversity was lower in snowy and white-fronted plovers (Table 5). The differences in diversity could be biased by the markers chosen, because 21 of the 26 microsatellite markers were developed in Kentish plovers, and the variability of microsatellite markers usually drops with evolutionary distance from their source species (Primmer *et al.* 2005, Dawson *et al.* 2005). However, heterozygosities of the four markers that were specifically developed for snowy plover (Funk *et al.* 2007) or the barn swallow (Primmer *et al.* 1995) were also higher in Kentish plovers than in snowy and white-fronted plovers. Furthermore, mitochondrial sequences in Kentish plovers were also more diverse than those in white-fronted and snowy plovers (Table 3), and this difference cannot be explained by any ascertainment bias of microsatellite markers.

The genetic impoverishment of two of the groups and its role in the speciation process warrants further investigation. Genetic bottlenecks enhance the effects of random genetic drift, which then may promote divergence between isolated populations. Selection may play an important role in population divergence, but whether genetic drift can play an important role in the process of speciation remains a topic of debate (Coyne & Orr 2004, Futuyama 2005, Price 2007). Relating the timing of the bottlenecks in snowy and white-fronted plovers with the divergence time of the species will be helpful in evaluating whether genetic bottlenecks and genetic drift played an important role in the process of population divergence in plover populations.

***4. Large panmictic populations***

The breeding populations of both snowy and Kentish plover have become increasingly fragmented, probably due to human alterations of their habitat. However, $F_{st}$ and $\Phi_{st}$ analyses did not indicate population structuring. This result suggests that there are no barriers for gene flow over large distances within the analysed subspecies. Also, mitochondrial haplotypes were not associated with geography in both *nivosus* and *alexandrinus* subspecies. A number of Kentish plovers from Spain and UAE shared the same haplotype, although these locations are separated by almost 6000 km. The Bayesian analyses did not find differences between microsatellite profiles of plovers within Eurasia or within America, and assigned all individuals from each subspecies into the same cluster. A lack of population structure has also been found in a number of other shorebird species (Ottvall *et al.* 2005, Oyler-McCance *et al.* 2005, Marthinsen *et al.* 2007). Funk *et al.* (2007) evaluated population and subspecies differentiation in snowy plovers and noted that, although there was support for genetic differentiation between the three American subspecies, *C. a. nivosus* populations from were not genetically differentiated over large distances. The *nivosus* sampling of Funk *et al.* (2007) was restricted to sites from the US, and our results indicate that Pacific snowy plovers breeding more than 1000 km further south in Mexico belong to this subspecies and that gene exchange with the northern populations is not restricted which is important for conservation management of *C. a. nivosus*. Note that the differences between the American subspecies in Funk *et al.* (2007) were much smaller than the differences between the intercontinental groups that have presented here.

The suggested phylogenetic relationships between snowy, Kentish and white-fronted plovers is consistent with the hypothesis that transitions between different breeding systems have frequently occurred in the phylogenetic history of shorebirds (Székely & Reynolds 1995). In shorebirds,

biparental care is considered to be the ancestral state and uniparental male care is rare among species of *Charadrii* (Székely & Reynolds 1995). Mitochondrial and microsatellite markers suggest that the snowy plover diverged from the common ancestor of Kentish and white-fronted plovers. However, snowy and Kentish plovers both have high levels of uniparental male care and polyandry, with 27–37% of the deserting females remating (Warriner *et al.* 1986, Székely & Williams 1995, Amat *et al.* 1999). In contrast, the white-fronted plover is considered to be monogamous, with biparental care and brood desertion by either sex never having been reported (Lloyd 2008). Hence the most parsimonious explanation for the current breeding system in white-fronted plover is that biparental care has evolved from an ancestral state of uniparental care.

Our main aim was to investigate the divergence between Eurasian and American populations, between which we discovered strong differentiation. However, it may be argued that our call to reconsider the taxonomy is not appropriate because we did not include samples from all six *Charadrius alexandrinus* subspecies. For a comprehensive phylogeographic analysis and a robust phylogeny of the entire superspecies more sampling will be necessary. However, the populations sampled in Eurasia all belong to the subspecies *C. a. alexandrinus*, which has the largest distribution range of all the *C. alexandrinus* subspecies, and the comparison of these populations with the most widely distributed subspecies in America, *C. a. nivosus*, showed strongly pronounced differences that warrant a reconsideration of the current classification of the Kentish plover as cosmopolitan species. As mentioned above, an investigation of subspecies differentiation in snowy plovers has been conducted previously (Funk *et al.* 2007). A further fine-scale phylogenetic analysis, including populations of red-capped plovers, Javan plovers and samples from the subspecies of *C. a. seebohmi* and *C. a. dealbatus* combined with a more distantly related outgroup, may reveal further cryptic species and will help us to better understand the phylogenetic history of this species complex.

We have shown that Kentish and snowy plovers can no longer be considered to comprise a single cosmopolitan species. The ‘cosmopolitan’ label often depends on conservative taxonomy that often does not hold when the phylogeography is investigated thoroughly (e.g. Klautau *et al.* 1999, Bleidorn *et al.* 2006). Since molecular techniques have become available many species that were classified based on morphological characters alone have been found to consist of multiple cryptic species (reviewed by Bickford *et al.* 2007). Among shorebirds, the other currently considered

cosmopolitan species, the black-winged stilt (Hayman *et al.* 1986, del Hoyo *et al.* 1996), shows a large number of different geographical morphotypes and could potentially harbour several further cryptic species.

To summarise, we found substantial genetic and phenotypic differences between Eurasian and American populations of *C. alexandrinus*. The genetic differences exceed the differences found between Kentish and white-fronted plovers, two recognised distinct species. We recommend that the systematic status and nomenclature of *C. alexandrinus* should be changed and that the snowy plover should be recognised as a distinct species.

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**Table 1** Biometrics of snowy and Kentish plovers. 40 males, 40 females and 16 chicks during the first 24 h after hatching were measured from each breeding populations. Mean (±SD) are given. Summary statistics of two-way ANOVAs for adults and one-way ANOVAs for chicks from minimum adequate models are presented on the right.

| | **snowy plover** | **Kentish plover** | | | | **Sex** | | | **Population** | | | **Sex*Population** | | | **Residual** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Ceuta, Mexico** | **Al Wathba, UAE** | **Doñana, Spain** | **Tuzla, Turkey** | **Miklapuszta, Hungary** | ***df*** | ***F*** | ***P*** | ***df*** | ***F*** | ***P*** | ***df*** | ***F*** | ***P*** | ***df*** |
| **adult males** | | | | | | **adults** | | | | | | | | | |
| tarsus length [mm] | 25.56 ± 0.67 | 29.15 ± 1.36 | 29.44 ± 0.94 | 29.22 ± 0.96 | 29.39 ± 1.32 | 1 | 43.5 | < 0.001 | 4 | 198.2 | < 0.001 | - | - | - | 394 |
| wing length [mm] | 108.59 ± 2.57 | 110.33 ± 3.83 | 112.31 ± 2.44 | 108.90 ± 3.54 | 111.45 ± 3.36 | 1 | 4.2 | 0.04 | 4 | 28.6 | < 0.001 | - | - | - | 394 |
| body mass [g] | 38.48 ± 2.72 | 36.95 ± 2.86 | 40.66 ± 2.43 | 40.76 ± 2.09 | 44.75 ± 3.00 | 1 | 1.8 | 0.19 | 4 | 108.5 | < 0.001 | 4 | 2.55 | 0.04 | 390 |
| **adult females** | | | | | | | | | | | | | | | |
| tarsus length [mm] | 24.78 ± 0.89 | 28.39 ± 1.15 | 28.56 ± 0.97 | 28.61 ± 1.07 | 28.85 ± 1.29 | | | | | | | | | | |
| wing length [mm] | 106.55 ± 3.47 | 109.25 ± 2.79 | 111.79 ± 3.18 | 108.89 ± 2.91 | 111.88 ± 2.91 | | | | | | | | | | |
| body mass [g] | 37.76 ± 2.48 | 37.17 ± 2.90 | 41.74 ± 2.07 | 42.22 ± 2.20 | 44.43 ± 2.70 | | | | | | | | | | |
| **Chicks** | | | | | | **chicks** | | | | | | | | | |
| tarsus length [mm] | 17.57 ± 0.60 | 18.31 ± 1.01 | 18.88 ± 0.46 | 19.24 ± 0.78 | 19.63 ± 1.25 | - | - | - | 4 | 13.9 | <0.001 | - | - | - | 75 |
| body mass [g] | 5.84 ± 0.46 | 6.44 ± 0.49 | 6.4 ± 0.60 | 6.33 ± 0.39 | 6.00 ± 0.41 | - | - | - | 4 | 5.0 | 0.001 | - | - | - | 75 |

**Table 2** Net sequence differences of mtDNA for different plover groupings. Differences are based on the 1281-bp combined mitochondrial sequence derived from i) control region, ii) *ATPase* subunit *6* and partial subunit 8 gene, and iii) partial *ND3* gene. Significance thresholds were adjusted using the sequential Bonferroni method.

| **Comparison** | **Average bp difference (%)** | ***P*** |
|---|---|---|
| Interspecies | | |
| Kentish vs snowy plover | 89.4 (7.0) | $< 0.001^{***}$ |
| Kentish vs white-fronted plover | 46.9 (3.7) | $< 0.001^{***}$ |
| snowy vs white-fronted plover | 101.4 (7.9) | $< 0.001^{***}$ |
| Interpopulation | | ns |
| Ceuta vs Salt Lake | 2.3 (0) | ns |
| Al Wathba vs Doñana | 5.7 (0) | ns |
| Al Wathba vs Tuzla | 4.3 (0) | ns |
| Al Wathba vs Kujalnik | 6.0 (0) | ns |
| Doñana vs Tuzla | 5.0 (0) | ns |
| Doñana vs Kujalnik | 6.2 (0) | ns |
| Tuzla vs Kujalnik | 4.8 (0) | ns |

*** significant at $P < 0.001$

**Table 3** Summary of genetic variation indices of three mtDNA markers for two snowy plover (*Charadrius alexandrinus nivosus*), one white-fronted plover (*Charadrius marginatus*) and four Kentish plover (*Charadrius alexandrinus alexandrinus*) populations. All mtDNA markers combined covered a total of 1281 bp mtDNA sequence.

| | | ***ATPase 6/8*** | | | ***ND3*** | | | **Control Region** | | | **All combined** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Population** | **N** | **h** | **s** | **π** | **h** | **s** | **π** | **h** | **s** | **π** | **h** | **s** | **π** |
| **snowy plover** | | | | | | | | | | | | | |
| Ceuta, Mexico | 8 | 0.00 | 0 | 0.00 | 0.00 | 0 | 0.00 | 0.86 | 5 | 2.36 | 0.86 | 5 | 2.36 |
| Salt Lake, Utah | 5 | 0.40 | 1 | 0.40 | 0.00 | 0 | 0.00 | 0.4 | 4 | 1.6 | 0.4 | 5 | 2.00 |
| **white-fronted plover** | | | | | | | | | | | | | |
| West coast, Madagascar | 4 | 0.00 | 0 | 0.00 | 0.67 | 1 | 0.67 | 0.5 | 1 | 0.5 | 0.83 | 2 | 1.17 |
| **Kentish plover** | | | | | | | | | | | | | |
| Al Wathba, UAE | 8 | 0.86 | 3 | 1.11 | 0.25 | 1 | 0.25 | 0.89 | 10 | 3.57 | 0.96 | 14 | 4.93 |
| Doñana, Spain | 9 | 0.42 | 2 | 0.44 | 0.22 | 1 | 0.22 | 0.92 | 6 | 2.78 | 0.97 | 9 | 3.44 |
| Tuzla, Turkey | 9 | 0.81 | 2 | 0.83 | 0.47 | 1 | 0.47 | 0.84 | 11 | 4.31 | 1.00 | 14 | 5.50 |
| Kujalnik, Ukraine | 10 | 0.64 | 3 | 0.76 | 0.53 | 1 | 0.53 | 0.93 | 12 | 3.76 | 0.98 | 16 | 5.04 |

*ATPase 6/8,* ATPase subunit 6 and partial ATPase subunit 8
*ND3,* partial ND3 gene for NADH dehydrogenase subunit 3
*h*, haplotype diversity
*s*, number of polymorphic sites
*π*, nucleotide diversity

**Table 4** $\Phi_{st}$ values for mitochondrial markers (above diagonal) and $F_{st}$ values for microsatellite markers (below diagonal) between seven plover populations. $\Phi_{st}$ and $F_{st}$ values are presented for comparisons between breeding populations from three groups: snowy plovers (populations from Ceuta, and Salt Lake), white-fronted plover (Madagascar), Kentish plovers (populations from Al Wathba, Doñana, Tuzla and Kujalnik). $\Phi_{st}$ and $F_{st}$ values were tested for significance using 110 permutations, and sequential Bonferroni correction was applied to account for multiple tests.

| | **Populations** | **mtDNA $\Phi_{st}$** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Ceuta | Salt Lake | Madagascar | Al Wathba | Doñana | Tuzla | Kujalnik |
| **Microsatellite** | Ceuta | - | 0.02 ns | 0.98 *** | 0.96 *** | 0.95 *** | 0.97 *** | 0.96 *** |
| | Salt Lake | -0.01 ns | - | 0.98 *** | 0.96 *** | 0.95 *** | 0.97 *** | 0.95 *** |
| | Madagascar | 0.61 *** | 0.63 *** | - | 0.92 *** | 0.90 *** | 0.94 *** | 0.91 *** |
| | Al Wathba | 0.36 *** | 0.28 *** | 0.33 *** | - | 0.09 ns | 0.01 ns | 0.17 ns |
| | Doñana | 0.34 *** | 0.26 *** | 0.32 *** | 0 ns | - | 0.09 ns | 0.13 ns |
| | Tuzla | 0.36 *** | 0.28 *** | 0.36 *** | 0.01 ns | 0.01 ns | - | 0.10 ns |
| | Kujalnik | 0.38 *** | 0.28 *** | 0.35 *** | 0 ns | 0 ns | 0 ns | - |

*ns*, $F_{st}$/ $\Phi_{st}$ is not significant

***, $F_{st}$/$\Phi_{st}$ is significant with $P < 0.00$

**Table 5** Allele sizes and genetic variation of two snowy plover, one white-fronted plover and four Kentish plover populations measured by 26 autosomal microsatellite loci.

| | snowy plover | | | | | | | | white-fronted plover | | | | Kentish plover | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ceuta | | | | Salt Lake | | | | Madagascar | | | | Al Wathba | | | | Doñana | | | | Tuzla | | | | Kujalnik | | | |
| Locus | *N* | *A* | $H_e$ | Allele range | *N* | *A* | $H_e$ | Allele range | *N* | *A* | $H_e$ | Allele range | *N* | *A* | $H_e$ | Allele range | *N* | *A* | $H_e$ | Allele range | *N* | *A* | $H_e$ | Allele range | *N* | *A* | $H_e$ | Allele range |
| $^{FAM}$C201$^{*}$ | 30 | 2 | 0.38 | 127-131 | 5 | 2 | 0.47 | 127-131 | 19 | 3 | 0.28 | 133-139 | 30 | 11 | 0.90 | 127-149 | 30 | 12 | 0.87 | 129-157 | 30 | 15 | 0.90 | 125-163 | 22 | 11 | 0.86 | 129-157 |
| $^{NED}$C203$^{*}$ | 30 | 6 | 0.57 | 175-191 | 5 | 3 | 0.64 | 181-191 | 19 | 1 | 0 | 179 | 30 | 14 | 0.82 | 179-204 | 30 | 10 | 0.85 | 179-199 | 30 | 12 | 0.82 | 179-208 | 21 | 10 | 0.81 | 178-201 |
| $^{FAM}$C204$^{*}$ | 30 | 7 | 0.73 | 199-213 | 4 | 4 | 0.75 | 199-207 | 19 | 8 | 0.81 | 195-211 | 29 | 15 | 0.89 | 187-237 | 29 | 16 | 0.91 | 187-229 | 30 | 18 | 0.89 | 187-239 | 22 | 12 | 0.89 | 195-231 |
| $^{HEX}$C205$^{*}$ | 30 | 2 | 0.36 | 179-181 | 4 | 2 | 0.43 | 179-181 | 19 | 4 | 0.29 | 177-193 | 29 | 8 | 0.86 | 177-195 | 29 | 9 | 0.87 | 177-195 | 29 | 10 | 0.87 | 167-195 | 22 | 8 | 0.84 | 177-195 |
| $^{FAM}$Calex-01$^{\dagger}$ | 30 | 1 | 0 | 239 | 5 | 1 | 0 | 239 | 19 | 1 | 0 | 239 | 29 | 9 | 0.74 | 241-257 | 30 | 10 | 0.72 | 219-257 | 30 | 10 | 0.80 | 239-259 | 22 | 11 | 0.85 | 239-259 |
| $^{PET}$Calex-02$^{\dagger}$ | 30 | 2 | 0.35 | 146-150 | 5 | 2 | 0.53 | 146-150 | 18 | 3 | 0.30 | 152-158 | 30 | 11 | 0.89 | 146-168 | 30 | 11 | 0.86 | 152-174 | 30 | 15 | 0.90 | 144-182 | 22 | 10 | 0.86 | 148-176 |
| $^{NED}$Calex-04$^{\dagger}$ | 30 | 2 | 0.36 | 213-215 | 5 | 2 | 0.36 | 213-215 | 19 | 4 | 0.29 | 211-227 | 30 | 8 | 0.85 | 211-229 | 29 | 9 | 0.86 | 211-229 | 30 | 10 | 0.87 | 201-229 | 22 | 8 | 0.84 | 211-229 |
| $^{FAM}$Calex-05$^{\dagger}$ | 30 | 3 | 0.37 | 185-187 | 5 | 4 | 0.53 | 185-188 | 19 | 1 | 0 | 189 | 30 | 7 | 0.73 | 187-193 | 30 | 6 | 0.62 | 187-192 | 30 | 6 | 0.72 | 185-192 | 22 | 6 | 0.61 | 187-192 |
| $^{HEX}$Calex-08$^{\dagger}$ | 30 | 2 | 0.03 | 226-228 | 5 | 1 | 0 | 228 | 19 | 1 | 0 | 224 | 30 | 5 | 0.70 | 222-230 | 30 | 5 | 0.65 | 223-230 | 30 | 5 | 0.72 | 222-230 | 22 | 4 | 0.72 | 224-230 |
| $^{HEX}$Calex-10$^{\dagger\S}$ | 30 | 3 | 0.42 | 214-220 | 5 | 2 | 0.53 | 214-216 | 19 | 2 | 0.27 | 203-205 | 25 | 3 | 0.46 | 201-205 | 26 | 3 | 0.57 | 201-205 | 25 | 3 | 0.60 | 201-205 | 19 | 2 | 0.42 | 203-205 |
| $^{NED}$Calex-11$^{\dagger}$ | 30 | 1 | 0 | 155 | 5 | 1 | 0 | 155 | 19 | 2 | 0.50 | 154-155 | 26 | 9 | 0.85 | 154-163 | 29 | 9 | 0.84 | 154-163 | 26 | 9 | 0.85 | 151-162 | 20 | 9 | 0.86 | 154-163 |
| $^{FAM}$Calex-12$^{\dagger}$ | 30 | 4 | 0.65 | 390-396 | 5 | 2 | 0.47 | 390-392 | 19 | 2 | 0.05 | 373-375 | 29 | 8 | 0.79 | 382-396 | 30 | 8 | 0.85 | 373-396 | 30 | 7 | 0.80 | 384-396 | 21 | 6 | 0.81 | 386-396 |
| $^{HEX}$Calex-14$^{\dagger}$ | 30 | 7 | 0.74 | 200-214 | 5 | 4 | 0.82 | 200-208 | 19 | 8 | 0.81 | 196-212 | 29 | 14 | 0.88 | 188-220 | 30 | 17 | 0.91 | 188-230 | 30 | 18 | 0.89 | 188-240 | 22 | 12 | 0.89 | 196-231 |
| $^{FAM}$Calex-18$^{\dagger}$ | 30 | 2 | 0.07 | 159-161 | 5 | 1 | 0 | 159 | 19 | 4 | 0.63 | 161-169 | 30 | 10 | 0.86 | 149-171 | 30 | 9 | 0.84 | 155-171 | 30 | 8 | 0.85 | 155-171 | 22 | 9 | 0.83 | 153-171 |
| $^{HEX}$Calex-19$^{\dagger}$ | 30 | 2 | 0.44 | 295-303 | 5 | 2 | 0.53 | 295-303 | 19 | 3 | 0.53 | 303-305 | 30 | 11 | 0.86 | 296-311 | 30 | 10 | 0.87 | 296-310 | 29 | 11 | 0.86 | 296-314 | 22 | 10 | 0.85 | 301-314 |
| $^{HEX}$Calex-22$^{\dagger}$ | 30 | 1 | 0 | 314 | 5 | 1 | 0 | 314 | 18 | 4 | 0.70 | 316-322 | 29 | 6 | 0.63 | 312-324 | 30 | 4 | 0.51 | 316-324 | 30 | 6 | 0.71 | 315-324 | 21 | 4 | 0.63 | 316-324 |
| $^{PET}$Calex-23$^{\dagger}$ | 30 | 5 | 0.55 | 232-246 | 5 | 3 | 0.64 | 236-246 | 19 | 1 | 0 | 234 | 30 | 15 | 0.84 | 234-259 | 30 | 11 | 0.85 | 234-254 | 30 | 12 | 0.83 | 229-263 | 22 | 10 | 0.80 | 229-256 |

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $^{FAM}$Calex-24$^{\dagger}$ | 30 | 1 | 0 | 112 | 5 | 1 | 0 | 112 | 19 | 1 | 0 | 86 | 30 | 7 | 0.62 | 86-114 | 30 | 5 | 0.60 | 86-112 | 30 | 5 | 0.59 | 86-114 | 22 | 4 | 0.64 | 86-112 |
| $^{NED}$Calex-28$^{\dagger}$ | | NA | NA | | | NA | NA | | 15 | 3 | 0.48 | 218-222 | 29 | 7 | 0.82 | 208-224 | 30 | 8 | 0.78 | 208-226 | 29 | 7 | 0.76 | 208-224 | 22 | 6 | 0.69 | 208-220 |
| $^{FAM}$Calex-32$^{\dagger}$ | 30 | 4 | 0.67 | 182-196 | 4 | 3 | 0.61 | 188-196 | 19 | 3 | 0.53 | 184-196 | 30 | 7 | 0.64 | 178-192 | 30 | 6 | 0.68 | 178-194 | 30 | 4 | 0.61 | 178-192 | 21 | 6 | 0.74 | 178-194 |
| $^{HEX}$Calex-35$^{\dagger}$ | 30 | 4 | 0.58 | 147-153 | 4 | 2 | 0.54 | 151-153 | 19 | 4 | 0.43 | 127-155 | 30 | 11 | 0.81 | 127-165 | 30 | 13 | 0.77 | 127-159 | 30 | 13 | 0.78 | 127-174 | 22 | 8 | 0.76 | 127-186 |
| $^{PET}$Calex-37$^{\dagger}$ | 30 | 1 | 0 | 178 | 5 | 1 | 0 | 178 | 19 | 2 | 0.19 | 174-178 | 29 | 9 | 0.81 | 166-198 | 29 | 10 | 0.84 | 166-196 | 29 | 13 | 0.88 | 166-202 | 21 | 11 | 0.88 | 166-204 |
| $^{HEX}$Calex-39$^{\dagger}$ | 30 | 4 | 0.33 | 106-112 | 5 | 1 | 0 | 110 | 19 | 4 | 0.15 | 135-145 | 30 | 19 | 0.94 | 114-169 | 30 | 11 | 0.86 | 124-145 | 30 | 20 | 0.86 | 120-171 | 22 | 16 | 0.88 | 124-155 |
| $^{FAM}$Calex-43$^{\dagger}$ | 30 | 5 | 0.56 | 389-395 | 5 | 3 | 0.51 | 389-393 | 19 | 7 | 0.79 | 388-404 | 30 | 21 | 0.94 | 374-434 | 29 | 19 | 0.94 | 373-423 | 30 | 19 | 0.94 | 374-408 | 22 | 17 | 0.95 | 374-404 |
| $^{FAM}$Calex-45$^{\dagger}$ | 30 | 2 | 0.07 | 254-256 | 5 | 1 | 0 | 256 | 19 | 2 | 0.05 | 258-262 | 30 | 11 | 0.82 | 253-292 | 30 | 10 | 0.82 | 256-284 | 28 | 13 | 0.88 | 253-284 | 22 | 12 | 0.87 | 253-284 |
| $^{NED}$Hru2$^{\ddagger}$ | 30 | 3 | 0.52 | 148-152 | 3 | 3 | 0.73 | 148-154 | 19 | 1 | 0 | 146 | 29 | 6 | 0.69 | 138-148 | 29 | 6 | 0.72 | 138-148 | 30 | 6 | 0.69 | 138-148 | 22 | 6 | 0.65 | 138-148 |
| All loci | 3.0 | | 0.35 | | 2.1 | | 0.36 | | 3.0 | | 0.31 | | 10.1 | | 0.79 | | 9.5 | | 0.79 | | 10.6 | | 0.81 | | 8.7 | | 0.78 | |



*FAM, HEX, NED, PET,* fluorescent labels of the forward primers; *N,* number of individuals genotyped; *A*, number of alleles found in population sample; *He,* Heterozygosity corrected for sample size according to Nei (1978); $^{*}$Primers for this locus originally developed for American snowy plovers (Funk *et al.* 2007); $^{\dagger}$ Primers for this locus originally developed for Eurasian Kentish plovers (Küpper *et al.* 2007);$^{\ddagger}$Primers for this locus originally developed for barn swallow (Primmer *et al.* 1995); $^{\S}$Locus not in Hardy-Weinberg equilibrium, but with homozygote excess when tested in 30 Kentish plovers breeding in Tuzla.

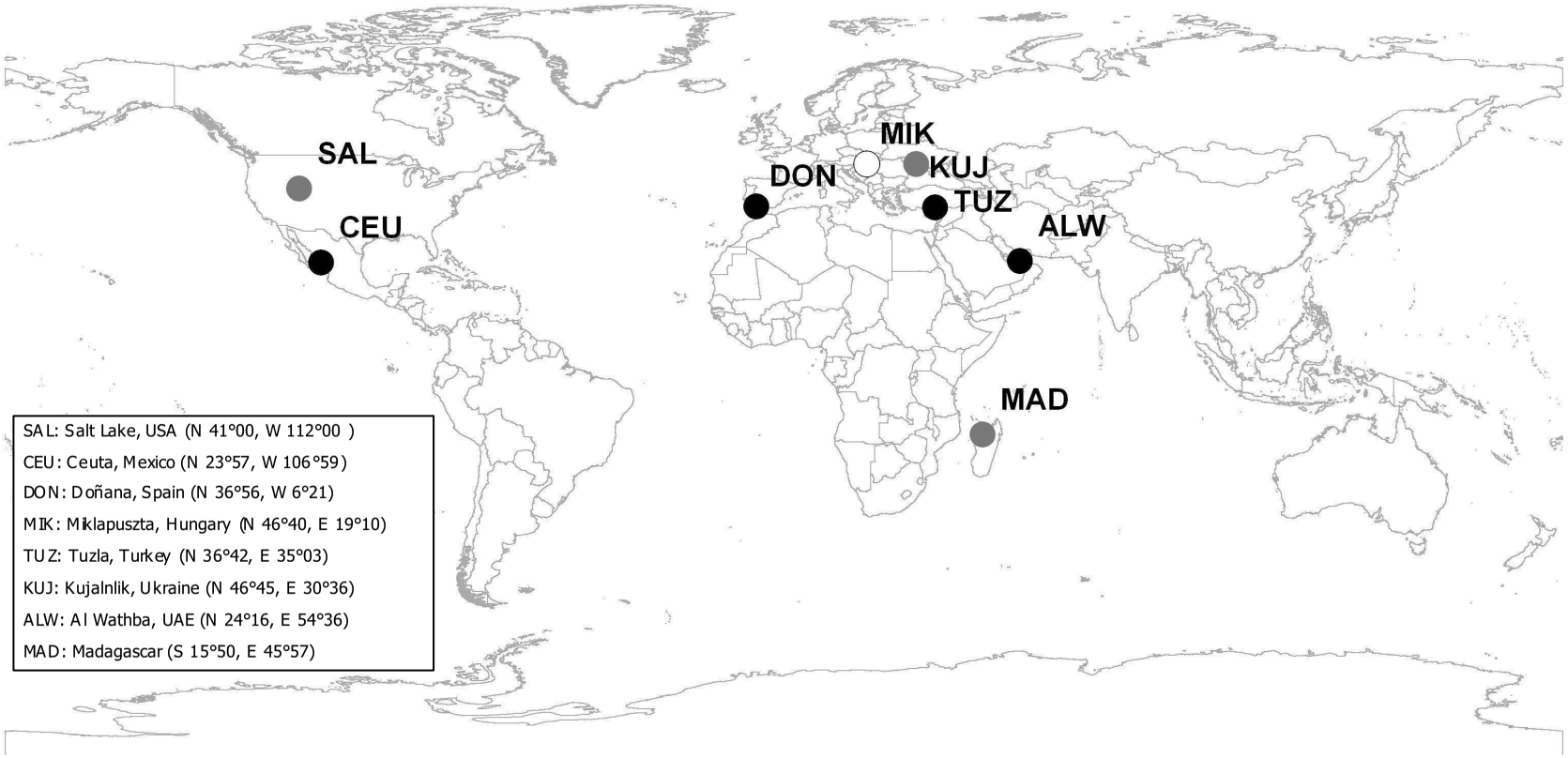


**Fig. 1** Sampling map for Kentish (Eurasia), snowy (America) and white-fronted plover (Africa) breeding populations. Black circles refer to locations where both DNA and biometric data were obtained, grey circles refer to locations where only DNA was sampled, the open circle indicates that only biometric measurements were taken. All sampled Kentish plovers belong to the subspecies *alexandrinus*, whilst all snowy plovers belong to the subspecies *nivosus*.

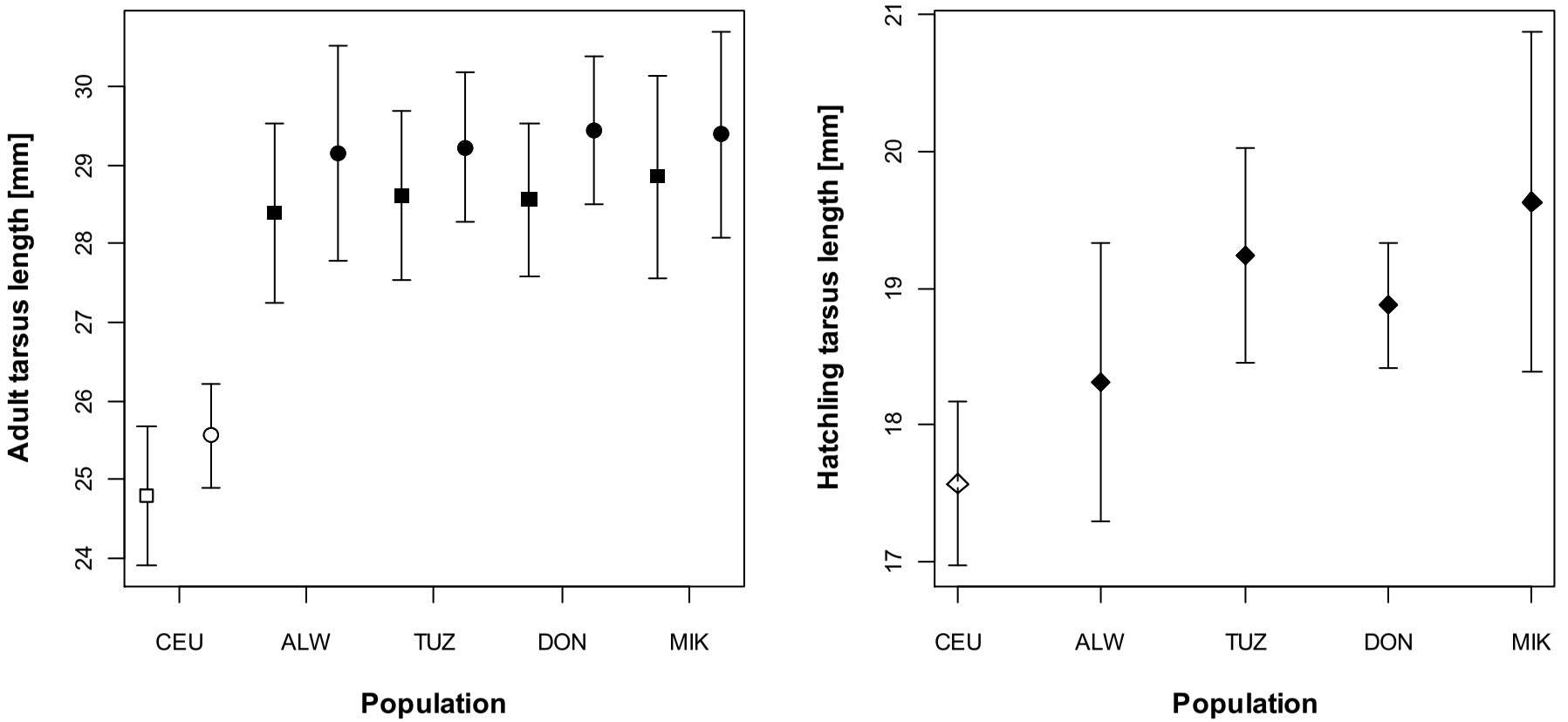


**Fig. 2** Population differences in tarsus length between adult and juvenile snowy and Kentish plovers. From each population 40 adult females (squares), 40 adult males (circles) and 16 chicks (diamonds) were included in the analyses. Open symbols refer to snowy plovers, filled symbols refer to Kentish plovers. Means (±SD) are presented.

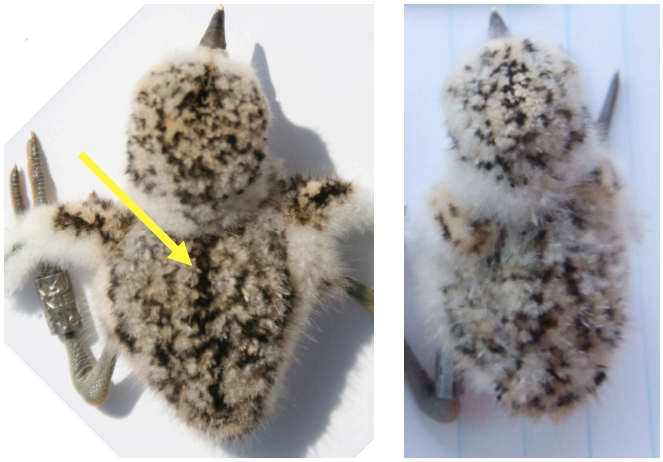

**Fig. 3** Downy chick plumage in Kentish and snowy plovers. Plumage of Kentish plover chicks (left) features a dark central stripe on the back (indicated by arrow), whilst this stripe is missing in snowy plover chicks (right). Both chicks were photographed a few hours after hatching. Kentish plover chick picture taken by T. Székely in Kujalnik, June 2007, snowy plover chick taken by C. Küpper in Ceuta, June 2007.

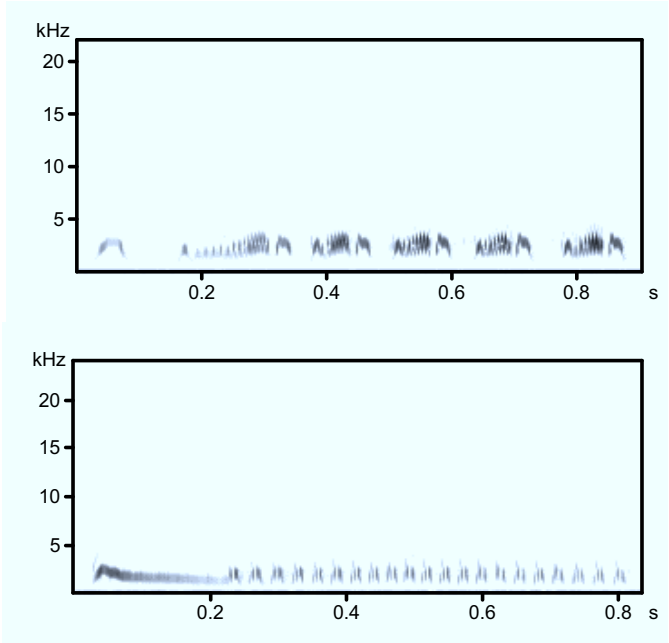


**Fig. 4** Male courtship calls of Kentish plovers sampled in France (top) and snowy plovers sampled in North Dakota, USA (bottom).

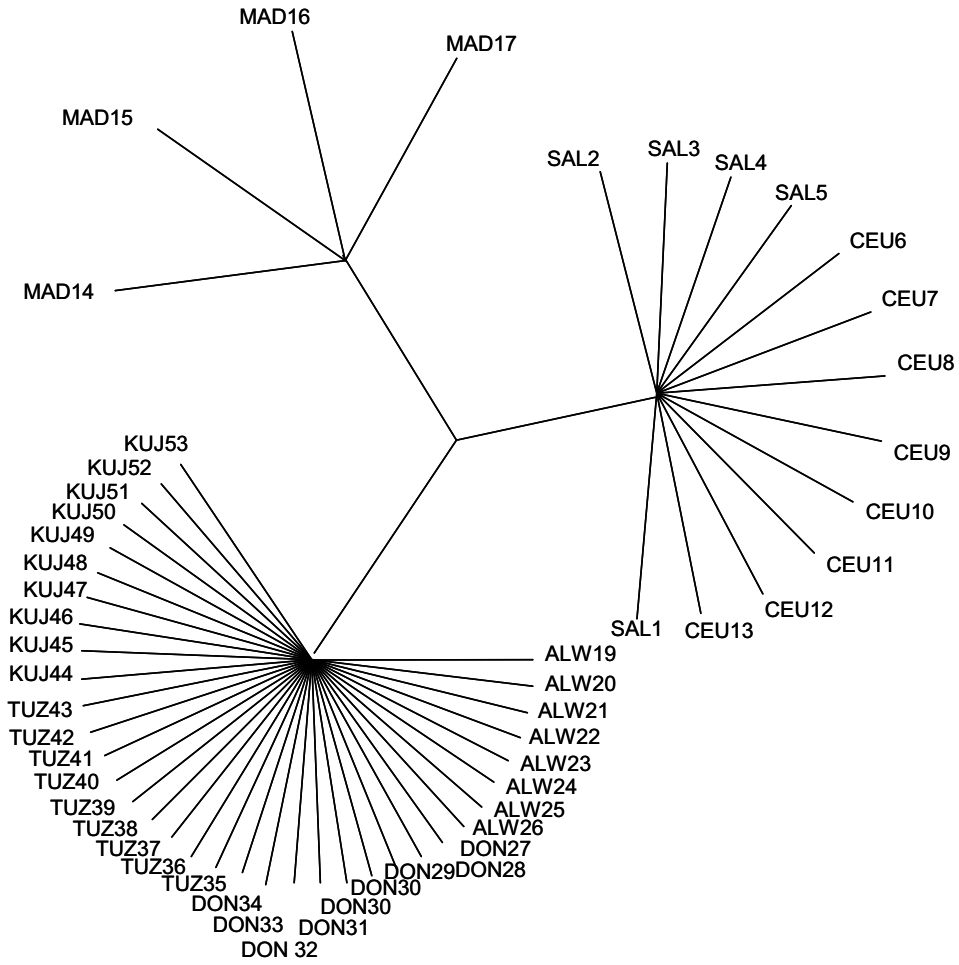


**Fig. 5** Unrooted strict consensus parsimony network for mitochondrial haplotypes of 53 white-fronted, snowy and Kentish plovers. The three major branches refer to white-fronted plover (4 MAD samples), snowy plover (5 SAL and 8 CEU samples) and Kentish plovers (10 KUJ, 9 TUZ, 9 DON and 8 ALW samples).

Chapter V

# Overdominance and underdominance of conserved microsatellite markers are associated with offspring survival in Kentish plover *Charadrius alexandrinus*

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**Manuscript**

**Contributions:**

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## Abstract

Heterozygosity-fitness correlations (HFCs) based on microsatellite markers have often been used as a proxy for inbreeding depression in natural populations, particularly when pedigrees are not available. However, although many positive HFCs have been reported, the correlation between marker-based heterozygosity estimators and the inbreeding coefficient is often weak or absent. These results have led to doubts about the utility of marker-based multilocus heterozygosity as estimator for genomewide heterozygosity. Instead, it has been argued that the observed HFCs are based on local or direct effects of microsatellite markers on fitness. Previous studies of HFCs have been mainly conducted in populations that are genetically impoverished, whilst investigations of genetically diverse, outbred populations are scarce. We investigated the relationship between marker-based heterozygosity and chick survival from hatching to fledgling, tarsus and body mass growth in a genetically diverse population of a small shorebird, the Kentish plover *Charadrius alexandrinus* using eleven unlinked conserved microsatellite markers. There was no overall genomewide effect of multilocus heterozygosity on morphological traits or chick survival. However, heterozygosity at three independent microsatellite loci was significantly correlated with chick survival. One of the HFCs revealed overdominance (heterozygosity is beneficial) whereas two showed underdominance (heterozygosity is detrimental). Our results allow us to reject the 'general effect hypothesis' in favour for 'local' or 'direct effect hypotheses' of marker-based heterozygosity, but further studies are needed to discriminate between the two latter hypotheses.

## Introduction

Genetic variability has important consequences for fitness. Many studies have reported a positive association between genetic diversity and fitness estimated by parasite resistance, reproductive success or survival in wild populations (Hansson *et al.* 2004, Piertney & Oliver 2006, Acevedo-Whitehouse *et al.* 2006, Kempenaers 2007). Often this positive relationship is (at least indirectly) associated with negative consequences of inbreeding. Inbreeding depression is caused by the loss of overdominance (heterozygosity has beneficial fitness effects) due to the loss of genomewide heterozygosity and the expression of deleterious recessive alleles (Hedrick & Kalinowski 2000, Keller & Waller 2002). However, to estimate inbreeding depression, information about the inbreeding coefficient of an individual is needed. Inbreeding coefficients have been traditionally estimated from pedigrees detailing the kin relationships between members of a population. However, for many populations long-term pedigrees that are necessary to estimate relatedness between population members cannot be obtained. Therefore genetic markers (particularly microsatellites) are frequently used to estimate heterozygosity and genetic diversity of individuals (Coulson *et al.* 1998, Amos *et al.* 2001, Hansson & Westerberg 2002, Aparacio *et al.* 2006).

Marker-based heterozygosity estimates were believed to provide a proxy for the inbreeding coefficient of an individual in the absence of pedigree data. However, there are problems with using microsatellite markers to investigate the effects of inbreeding in natural populations. First, genetic diversity is often estimated by a small number (< 15) of microsatellite markers, but the correlation between multilocus heterozygosity (MLH) and the inbreeding coefficient is often low even when more than 100 microsatellite markers are used (Slate *et al.* 2004). Second, inbreeding is relatively rare in most natural population and the variation in the inbreeding coefficient among individuals may not be enough to explain the observed associations between marker-based estimates of heterozygosity and fitness (Hansson & Westerberg 2008). Third, a publication bias towards significant positive results of heterozygosity-fitness correlations (HFCs) can hinder a better understanding about marker-based heterozygosity in regards to fitness (Coltman & Slate 2003). For example, theory predicts that at least for some regions of the genome not only overdominance (causing balancing selection), but also underdominance (causing disruptive selection) effects should exist. However, there are few studies reporting negative relationships between

marker-based heterozygosity and fitness (but see Lee *et al.* 2002, Lieutenant-Gosselin & Bernatchez 2006). Moreover, despite its frequently shown genetic costs inbreeding may not only have negative effects on fitness, but could increase cooperation between individuals, for instance, in the context of parental care it may lead to better coordination of care between parents and higher offspring survival (Houston *et al.* 2005, Kokko & Ots 2006, Thünken *et al.* 2007).

Several ways have been proposed to tackle some of the caveats above. First, new algorithms for MLH estimators have been developed which are better correlated with the inbreeding coefficient (Coulson *et al.* 1998, Amos *et al.* 2001, Aparicio *et al.* 2006). These improved MLH estimators take into account the special mutation process in microsatellites ($d^2$, Coulson *et al.* 1998) and the differential information harboured by different alleles ('internal relatedness', Amos *et al.* 2001), or loci ('homozygosity by loci', Aparicio *et al.* 2006). The newly derived MLH estimators are usually highly correlated to uncorrected MLH, but may perform better than the original measure when certain criteria of the markers are met (Aparicio *et al.* 2006).

The second line of research has questioned the generality of the MLH approach with its focus on genomewide effects and has focused on associations of a number of microsatellite markers that are presumably associated to fitness loci (Hansson *et al.* 2004, Markert *et al.* 2004, Lieutenant-Gosselin & Bernatchez 2006, Hansson & Westerberg 2008). This is based on the assumptions that heterozygosity in microsatellite markers is either directly responsible for variation in fitness, for example, when the microsatellite itself has a functional role ('direct effect') or, indirectly, through high linkage disequilibrium between markers and loci associated with functional effects ('local effect'). Support for the latter has come from studies on newly founded or island populations where high linkage disequlibrium is expected (Hansson *et al.* 2004, Markert *et al.* 2004), and theoretical models (Hansson & Westerberg 2008).

Microsatellite markers have been initially assumed to be neutral markers, although the evidence is growing that this may not always the case. Microsatellites were embedded in expressed genes, where a mutation could cause a deleterious frameshift of the reading frame if the repeat unit of the microsatellite is not a multiple of three (Metzgar *et al.* 2000, Tóth *et al.* 2002). Other microsatellites are involved in important structural and metabolic DNA processes such as chromatin organisation, regulation of gene activity,

replication and recombination (Li *et al.* 2002, 2004). The recent increase of sequence information for many taxa has made it feasible to map the location of markers on available chromosomal maps of related taxa and to explore the degree of conservation of these markers (Dawson *et al.* 2006, Küpper *et al.*, ms in preparation). Indeed, MLH at seven conserved microsatellite markers located in genes predicted parasite load measured by lungworm *Protostrongylus* spp. abundance in a population of wild bighorn sheep *Ovis canadensis*, but no association was found between MLH at eight putative neutral markers and lungworm abundance (Luikart *et al.* 2008). Furthermore in this study single locus heterozygosity (SLH) at three of the conserved markers was directly associated with parasite load. The degree of preservation of microsatellite markers across taxa may indicate local or direct functional effects. Particularly, microsatellite markers that have a wide cross-amplification range (Primmer *et al.* 2005, Dawson *et al.* 2005, Küpper *et al.*, ms in preparation) may be associated with fitness effects. In support, a number of studies reported significant correlations between SLH of markers with wide cross-amplification range and fitness (Hansson *et al.* 2001, Acevedo-Whitehouse *et al.* 2006).

We examined heterozygosity and offspring fitness in a small shorebird, the Kentish plover *Charadrius alexandrinus*. Kentish plovers nest on the ground and produce small clutches containing usually three eggs (Cramp & Simmons 1983). The chicks are precocial, i.e. they leave the nest scrape a few hours after hatching, and are led by their parents to the brood territory where they feed for themselves. Kentish plover young are highly suitable to study beneficial and adverse genetic effects because, as in other shorebirds, the young are exposed to a hostile environment immediately after hatching: their thermoregulation is still not fully developed and, although the parents give alarm calls when predators approach the family, the chicks have to fend off predators and attacks by conspecifics themselves. The costs of these attacks can be substantial, because chicks are injured and/or killed before fledging (Székely & Cuthill 1999). These challenges together with the fluctuating environment of temporal salt lakes which provide the breeding grounds, may put the genetic outfit of chicks under scrutiny, with selection eliminating detrimental allelic combinations.

We investigated HFCs in Kentish plover young. We examined heterozygosity at eleven conserved microsatellite loci and its relationship to three fitness measures: length of survival, tarsus and body mass growth. We asked whether genomewide or local/direct

heterozygosity effects predict survival of Kentish plover young best using different estimators for MLH and SLH. Fitness is not only dependent on genes, but also on environmental conditions and stochastic variables that can introduce substantial statistical noise. Therefore, in our models we controlled for previously identified environmental variables that influence chick survival (Székely & Williams 1995, Székely & Cuthill 1999, Székely *et al.* 2004).

## Material and Methods

### *Study population*

We studied a breeding population of Kentish plovers in the saltmarsh of Tuzla, Turkey (36°42 N, 35°03 W, details in Székely *et al.* 1999, Székely & Cuthill 2000, Kosztolányi *et al.* 2006). About 1000 pairs of Kentish plovers breed around a temporary salt lake at Tuzla, and a substantial proportion of adults and chicks was colour marked every year. The population harbours a large amount of genetic diversity with 53 (75 %) of 71 microsatellite markers tested being polymorphic (Küpper *et al.* 2007, ms & ms2 in preparation). The Tuzla breeding population is a part of a metapopulation that expands from the Atlantic coast to the Persian Gulf (Küpper *et al.*, ms2 in preparation). However, inbreeding may occur within a breeding site and extra-pair fertilisations that occur in low frequency in Tuzla appear to have an adaptive role in avoiding inbreeding depression (Blomqvist *et al.* 2002, Küpper *et al.* 2004).

### *Field methods*

Fieldwork was carried out from April to July between 1997 and 2000. We trapped adult plovers that were incubating on the nest or tending chicks and individually marked them with a metal and three colour rings (Székely *et al.* 2008). A small blood sample of approximately 20-50 µl was taken from the brachial vein for subsequent genotyping. Chicks were either ringed 0-2 days after hatching in the vicinity of the nest scrape or, for those from unknown nests, at varying ages during opportunistic encounters in the field. We marked chicks with a metal ring and a single colour ring indicating the brood identity. We measured tarsus length (to the nearest 0.1 mm) and body mass (to the nearest 0.1 g) at the first encounter and during subsequent recaptures. The age of chicks from unknown nest locations was estimated based on their mean tarsus length at capture (see Székely & Cuthill 1999). The age formula for the present data set (Age [days] = 2.48 x Tarsus length at capture [mm] – 47.3) was based on 306 chicks from known nests that were ringed a few hours after hatching assuming linear tarsus growth between

the age of 0 and 25 days. A small blood sample of approximately 20 µl was taken from the chick's tarsal vein.

Kentish plovers breed in open sparsely vegetated habitats which makes it feasible to follow families and record individual chick survival. Families were visited on average every other day until the age of 25 days at which we assumed the chicks fledge (cf Székely & Cuthill 1999). At each visit we noted the number of chicks, identity of the attending parent(s) (Székely & Cuthill 1999). If chick(s) had perished we took the date of the first observation without the chick as point of death for the missing chick. Chicks were regularly recaptured to identify survivors, measure tarsus length and body mass. If chicks of the same family had died at different ages and the order of death could not be determined from recapturing data we used the first observation when both chicks were missing as date of death for the chicks in question. Predation on chicks was rarely observed, however, we are confident the disappeared chicks died, because we made extensive searches for each missing chick.

***Molecular methods***

The details for DNA extraction and genotyping are specified elsewhere (Küpper *et al.* 2004, ms2 in preparation). In brief, DNA was extracted either according to an adapted phenol chloroform (Krokene *et al.* 1995) or ammonium acetate method (Nicholls *et al.* 2000). Chicks were genotyped at eleven conserved microsatellite markers *(Calex-01, -04, -05, -08, -12, -14, -18, -19, -22* and *-23,* Küpper *et al.* 2007*)* that were part of two multiplex reactions. We defined the marker as conserved if we could map it to a unique location on the chicken genome. The details of the mapping and the locations of these markers which could be assigned to seven different chicken chromosomes are described elsewhere (Küpper *et al.*, ms in preparation). Two of the conserved *Calex* markers described in this manuscript (*Calex-07* and *-17*) were omitted for the present study because they could not be fitted into the two multiplex reactions. We visualised the multiplex products using an ABI 3730 capillary sequencer and assigned genotypes for each individual using GENEMAPPER version 3.7 (Applied Biosystems). Only individuals that had been successfully genotyped at all loci (n = 385) were included into the heterozygosity-fitness analyses.

***Statistical procedures***

We used genotypes of 339 presumably unrelated breeding adults to determine whether microsatellite markers were in linkage disequilibrium and to test for neutrality at each locus. Heterozygosity and null allele frequency estimates were calculated using CERVUS version 2.0 (Marshall *et al.* 1998). Independence of heterozygosity estimates at each locus was tested in two ways. First, markers were tested pairwise using the linkage test implemented in GENEPOP version 4 (Raymond & Rousset 1995) using the following Markov Chain parameters: 100,000 dememorisation runs and 1,000 batches each with 20,000 iterations. Second, we tested whether heterozygosities at different loci were correlated with each other by calculating Spearman's *r* for each pairwise test. Finally, to test whether allele frequencies at any marker deviated significantly from frequencies predicted by Hardy-Weinberg equilibrium we conducted heterozygote excess and deficit tests in GENEPOP version 4.

We explored the effects of heterozygosity on chick survival and two characteristics of chick development: tarsus and body mass growth using mixed effects models. We investigated these response variables using both single and multilocus estimators for heterozygosity (explanatory variables). First, to examine SLH effects we fitted environmental variables and each microsatellite locus as fixed factors into the mixed model. Second, we prepared models for each of three MLH estimators: uncorrected average heterozygosity (all loci and alleles are equally weighed), 'internal relatedness' (Amos *et al.* 2001) and 'homozygosity by loci' (Aparacio *et al.* 2006). 'Internal relatedness' and 'homozygosity by loci' are two estimators that have been considered as superior over uncorrected average heterozygosity when increased homozygosity reflects a higher inbreeding coefficient. Internal relatedness is calculated by giving differential weights to different allele combinations according to their frequencies. Homozygosity involving rare alleles at a given locus will affect the estimator stronger than homozygosity involving frequent alleles. The estimator 'homozygosity by loci' is calculated by weighing loci according to their allelic variability. Homozygosity at more variable loci will affect the estimate stronger than homozygosity at less variable loci. All estimates of heterozygosities were calculated using the IRmacroN4 developed by W Amos (available from http://www.zoo.cam.ac.uk/zoostaff/amos/IRmacroN4.xls).

We tested the association between microsatellite heterozygosity and offspring survival using mixed effect Cox regression implemented in the R package 'kinship' (Therneau

2007). Death of the chicks was considered as the terminal event. Chicks that reached an age of 25 days or went missing together with their parents before they reached that age were included as censored data points and we used their last observation as final age.

In each model we statistically controlled for the potential effects of environmental factors that have been shown to influence Kentish plover chick survival: *Hatch date*, late hatched broods survived worse than early hatched broods probably due to deteriorating environmental conditions when the lake dries out (Székely & Cuthill 1999); *Site quality*, a binary variable based on the observations that broods that hatched close to the village survived worse and were considered to have poorer territories than broods that hatched further away from the village (Székely & Cuthill 1999); *Chick sex*, broods with female-biased chick ratios hatched later and survived worse than broods with male-based chick ratios (Székely *et al.* 2004); and *Length of biparental care* for the social brood since broods that are attended by both parents have higher fledging success than broods attended by only one parent (Székely & Williams 1995). Environmental factors and the heterozygosity estimators were included as fixed factors, and family identity was included as random factor.

Experimental manipulations of parents or broods were carried out between 1997 and 1999, although these manipulations did not influence survival or behaviour of families (Kosztolányi *et al.* 2006, 2007). Two of the genotyped chicks were naturally adopted by foster families and a total of 165 genotyped chicks (25 in 1998, 140 in 1999) were swapped between families within the first five days after hatching (e.g. Székely & Cuthill 1999, 2000). Offspring survival varied strongly between families. Conservatively, we run two sets of models that differed in the random factor 'family identity'. In the first set of models we considered 'biological family' (unmanipulated original nest in which the chick had hatched) as family identity. In the second set of models we fitted the 'social family' (the family in which a chick was last seen) as family identity.

To examine the influence of microsatellite heterozygosity on growth we used mixed effect models implemented in the R package 'nlme' (Pinheiro & Bates 2000). The residuals from the either tarsus or mass growth curve (see below) were taken as response variable. We only used chicks that had been ringed in their nest scrape for this analysis. Growth curves were calculated from measurements of chicks with known age

that were ringed in the nest scrape and recaptured before fledging (n = 473 measurements). Tarsus growth appeared to be linear up to age of 25 days (linear regression model: 0.40 x [age in days] + 19.06, $p < 0.001$, residual standard error ($SE_{Residual}$) = 1.05, df = 460). The relationship for body mass growth, however, appeared to be non-linear, and in addition, plover chicks, similar to other precocial chicks, lost mass during their first few days of life (C Küpper & T Székely, unpublished data). Since weight loss is not accounted for in traditional growth models, we explored five body mass growth models: linear growth (0.70 x [age in days] + 5.93, $p < 0.001$, $SE_{Residual}$ = 2.08, df = 471); Gompertz ($474.1 \times e^{-4.35 \times e^{-0.98 \times [\text{age in days}]}}$, $p < 0.001$, $SE_{Residual}$ = 1.95, df = 470), logistic ($56.52/(1 + 26.82 \times e^{-12.68 \times [\text{age in days}]})$, $p < 0.001$, $SE_{Residual}$ = 1.94, df = 470), von-Bertalanffy ($-3.76 - (-3.76 - 6.13) \times e^{0.05 \times [\text{age in days}]}$, $p < 0.001$, $SE_{Residual}$ = 1.95, df = 470); polynomial growth model ($41.59 \times e^{-0.18 \times [\text{age in days}]} + (41.59/(1 + 1.54 \times e^{-0.03 \times [\text{age in days}]}) + 41.59$, $p < 0.001$, $SE_{Residual}$ = 1.87, df = 469). The polynomial model had the best fit (i.e. the lowest $SE_{Residual}$) and consequently was chosen as most appropriate overall growth model for body mass. Fixed and random factors of the mixed models were fitted as for chick survival models (see above).

Testing a given set of hypotheses using the same data set requires adjustment for multiple testing because the false discovery rate (p-value is lower than significance threshold $\alpha$ by chance) increases with each additional test carried out. Traditional methods such as standard and sequential Bonferroni method (Rice 1989) lower $\alpha$ with each further test carried out. However, these methods have the disadvantage that the type II error rate, the rejection of the alternative hypothesis when it is true, increases disproportionally (Nakagawa 2004, Garcia 2004). This is a particular problem when several tests produce p-values that are smaller than 0.05, but due to adopted significance thresholds none of the test results is accepted as significant. The q-value provides a more powerful solution to control for false discoveries when multiple independent tests are carried out (Storey 2002). q-values incorporate information contained in the sample of observed p-values to determine the rejection threshold. For their calculation the distribution of observed p-values in the sample is compared to a uniform 'null' distribution of p-values which is expected when the null hypothesis is true for all tests (Storey 2002). q-values provide the probability that an observed p-value is the result of false discovery caused by multiple testing and their interpretation is analogous to p-values. We calculated q-values for independent tests (linkage tests and single loci comparisons) using the R package 'qvalue' (Dabney & Storey 2007). We considered

statistical tests as significant when both, p- and q-value were smaller than 0.05 and as marginally significant when both p- and q-vales where smaller than 0.1.

All statistical analyses were conducted in R version 2.6.2 (R Development Core Team 2008). Varying numbers of chicks in different models reflect limited data availability for some environmental explanatory variables. For the HFCs we only report the model statistics for the parameter of interest, the heterozygosity estimator. Full statistics of the models are given as supplementary material.

## Results

### *Linkage disequilibrium and Hardy-Weinberg equilibrium*

The eleven conserved microsatellite loci were highly polymorphic in the sample of adult breeders. The mean observed heterozygosity was 0.80 ranging from 0.66 at *Calex-22* to 0.89 at *Calex-14*. The mean estimate (± SE) of the proportion of null alleles was -0.002 ± 0.003. Four of the 55 conducted tests for linkage disequilibrium produced p-values < 0.05. However, after correcting for multiple tests, none of the tests was significant (all q-values > 0.15). Similarly, there was no significant association between heterozygosity from pairwise locus tests (three of the conducted tests with p-values < 0.05, but all q–values > 0.45). Finally, there was no evidence for balancing or directional selection on single markers since all conserved microsatellite loci conformed to Hardy-Weinberg equilibrium (all p-values larger than 0.17).

### *Chick survival*

Of 385 chicks with complete genotypes the fate of 184 chicks was known as fledged or died: 100 chicks were observed until an age of 25 days, 76 chicks were confirmed to have died before fledging (mean age ± SE = 5.7 days ± 0.5) and eight chicks whose fates had not been determined in the hatching season were recaptured as adult breeders in consecutive years (and hence classified as fledged). 201 chicks with unknown fates disappeared at a mean age of 6.2 days ± 0.7.

We found antagonistic trends in the effects of SLH. On the one hand, two loci *Calex-19* and *Calex-23* were associated with underdominance in models with social family as random factor and marginally associated in models with biological family as random factor (all p- and q- values ≤ 0.06) suggesting that homozygous chicks survived longer than heterozygous chicks (Table 1). The death hazard increased more than 200 % for

chicks being heterozygous at either of these loci. On the other hand, *Calex-18* showed significant overdominance in models with biological family as random factor and was marginally associated with overdominance in models with social family as random factor suggesting that chicks that were heterozygous at this locus survived significantly longer than chicks that were homozygous (Table 1). The hazard of death before fledging was almost reduced by 50 % for *Calex-18*-heterozygous chicks in comparison with homozygous chicks (Table 1). At one further locus (*Calex-22*) there was a marginally significant trend and chicks with homozygous genotypes appeared to survive better than heterozygous chicks in models with biological family as random factor, but not in models with social family as random factors (Table 1). Seven conserved loci showed no significant relationship between heterozygosity and chick survival (Table 1).

Chick survival was not associated with any of the three MLH estimators (uncorrected average heterozygosity, internal relatedness or homozygosity by loci, Table 1).

***Growth***

None of the heterozygosity estimators (SLH or MLH) predict tarsus or body mass growth significantly after controlling for multiple testing (Tables 2 & 3).

## Discussion

***Heterozygosity and survival***

We have shown that marker-based heterozygosity is correlated with survival of precocial offspring involving both over- and underdominance effects, although both effects were only marginally significant. Recent years have seen an increasing interest in the effects of marker-based heterozygosity on fitness, however, one of the most important component on fitness, survival has rarely been measured (David 1998). Using fine-scale observational and recapture data we show that heterozygosity in certain genomic regions affects the length of survival of Kentish plover chicks until fledging We are aware of only one further published study that investigated the relationship between marker-based heterozygosity and life span. In Darwin's ground finches *Geospiza fortis* more heterozygous adults live longer than homozygous adults, however, there was no association between heterozygosity and life span in the closely related *G. scandens* (Markert *et al.* 2004). Associations of MLH with offspring survival have been only reported from a handful of other vertebrate populations before. For instance, in great reed warblers *Acrocephalus arundinaceus* more heterozygous siblings are more

likely to be recruited into the breeding population than their more homozygous siblings (Hansson *et al.* 2001, 2004). Genetic diversity also predicted survival of juvenile alpine marmots *Marmota marmota* with more heterozygous juveniles being more likely to become yearlings than more homozygous juveniles and this effect was particularly strong in years with poor environmental conditions (Da Silva *et al.* 2006). In California sea lion *Zalophus californianus* more heterozygous pups survived better and were less affected by hookworms than more homozygous pups (Acevedo-Whitehouse *et al.* 2006).

***Underdominance***

Three conserved loci were involved in generating the HFCs in our study. However, in Kentish plovers heterozygosity was not purely beneficial on offspring survival as most other studies have reported. After controlling for environmental factors that influence chick survival we found both overdominance (at one locus) and underdominance effects (at two loci) showing significant HFCs. Few studies have reported negative heterozygosity effects associated with molecular markers. Lee *et al.* (2002) found that male moorhen *Gallinula chloropus* chicks heterozygous at the sex-chromosomal *CHD* gene had higher mortality than homozygous ones. In threespine sticklebacks *Gasterosteus aculeatus* MLH estimated from microsatellite loci was negatively associated with male mating success, whilst it was positively associated with other fitness measures such as territoriality and morphological traits (Lieutenant-Gosselin & Bernatchez 2006). This lack of underdominance reports may be explained by a publication bias (see Coltman & Slate 2003) and/or by a study bias: HFCs are often studied in island, founder or fragmented populations were genetic diversity is expected to be low (Markert *et al.* 2004, Da Silva *et al.* 2006, Luikart *et al.* 2008, Hansson *et al.* 2001, 2004, Jensen *et al.* 2007, Brouwer *et al.* 2007), whereas we examined heterozygosity in a genetically diverse, presumably outbred population. In genetically depleted population positive heterozygosity effects may be predominant and have a larger impact on survival than negative heterozygosity effects.

One explanation for the occurrence of underdominance could be the presence of null alleles at loci with negative HFCs. In this case heterozygotes would be falsely classified as homozygotes. However, this is unlikely to be the case in our study since the estimates for null alleles were small at all eleven conserved loci used (all $< 0.02$). Underdominance may occur when two populations are mixed and outbreeding then

causes locally adapted gene groups to break up. Currently there is no support for recent population admixture in the Kentish plover since the Turkish population appears to be part of a large genetically undifferentiated metapopulation (Küpper *et al.*, ms2 in preparation). Therefore outbreeding depression is unlikely to explain the observed underdominance patterns. Another explanation involves detrimental mutations at underdominant loci. Microsatellite variability at some loci that are located in expressed genomic regions may be detrimental if they cause frameshift changes which then leads to a knockout of the gene-function or heterozygosity. The conserved microsatellite markers were all mapped to chicken chromosomes and none of the markers was found to be located in chicken exons (Küpper *et al.*, ms in preparation, C Küpper, unpublished data), however, variability may also affect non-coding microsatellites with regulatory function or roles in DNA organisation (Li *et al.* 2002). Another explanation for underdominance is disruptive selection. Disruptive selection often favours extreme phenotypes (Rueffler *et al.* 2006). If heterozygosity leads to intermediate phenotypes, heterozygous individuals may have inferior survival than homozygous individuals. Disruptive selection often promotes genetic and phenotypic variation and strikingly, Eurasian Kentish plover populations harbour an unusually high genetic and behavioural diversity (this study, Küpper *et al.*, ms & ms2 in preparation, Amat *et al.* 1999, Székely *et al.* 2006). Further studies on outbred populations are needed to determine the importance of underdominance.

***General, local or direct effects of marker-based heterozygosities***

MLH was unrelated to inbreeding depression, nor did it have an overall general effect on fitness in this population for two reasons. First, there was no relationship between MLH and any of the three fitness measures. Second, there was no general positive (or negative) direction of heterozygosity across independent markers, but rather antagonistic over- and underdominance effects at different loci. Two alternative hypotheses, the 'local effect hypothesis' and 'direct effect hypothesis' (Hansson & Westerberg 2002) remain to explain the occurrence of the HFCs. To discriminate between the remaining hypotheses a detailed examination of the surrounding genomic regions is necessary. Presently the local effects, an association between microsatellite markers and fitness genes appears to be the more likely explanation. It is unlikely that the microsatellites were directly transcribed since they were not located in chicken exons (C Küpper, unpublished data). Synteny has been found in chromosome organisation between other groups of Neognath birds and chicken (Dawson *et al.* 2007)

and therefore it is unlikely that the used microsatellite markers are located within a gene in Kentish plover, but not in chicken. Allele frequencies and heterozygosities at all markers including those with overdominant and underdominant relationships with chick survival did not deviate from Hardy-Weinberg equilibrium, which may suggest that the markers were rather weakly linked to other loci with direct fitness effects and selection can be better traced at the fitness loci directly.

Inbreeding, founder effects and population admixture facilitate linkage disequilibrium (Slatkin 2008). We argue that linkage disequilibrium is propably low in the Tuzla breeding population for three reasons. First, as we have argued above there is no evidence for population admixture. Second, inbreeding is rare in the Turkish Kentish plover population (Blomqvist *et al.* 2002). Third, there are no signs that the population was recently founded or went through a bottleneck because the population is genetically highly diverse (Küpper *et al.*, ms2 in preparation). Pairwise linkage tests of 36 microsatellite markers tested in this population using 42 adults were all nonsignificant (Küpper *et al.* 2007), suggesting that linkage disequilibrium between these loci is low in this population.

Although the results are in the line with the 'local effect hypothesis', we cannot rule out the 'direct effect hypothesis' completely. Microsatellites are usually assumed to be neutral, however, recently doubts have been raised about the generality of this assumption because many microsatellites have been found to be located in functional genomic regions (Li *et al.* 2002, 2004). About 10 % of microsatellites are found in expressed regions in vertebrates (Li *et al.* 2004). Still many researchers usually disregard the possibility of direct marker effects on fitness and interpret lack of a general effect from HFCs as sufficient support for the 'local effect hypothesis'. Although the microsatellite markers used in this study were not directly associated with genes, we cannot exclude the possibility that the conserved microsatellites are involved in other functions. Their preservation across a broad range of taxa indicates a functional role or at least close association with a functional locus. Many studies make use of cross-amplifying microsatellite markers originally developed in different species, and the constrained evolution of such microsatellites (or at least the genomic region into which the microsatellite is embedded) suggests some form of functionality. For the ultimate proof or disproof of 'local and direct effect hypotheses' candidate genes

associated with the markers need to be identified and the genotypes of these and the markers need to be compared.

***Comparisons between heterozygosity effects on morphological and life history traits***

Neither SLHs nor MLH were associated with morphological traits of chick fitness, tarsus and body mass growth. This is consistent with the results of a recent meta-analysis showing that heterozygosity has a larger effect sizes for life history traits than for morphological traits (Coltman & Slate 2003). Coltman & Slate (2003) argued their measure of heterozygosity, MLH, could be a proxy for inbreeding depression and hence inbreeding depression would act stronger on life history traits than on morphological traits. This is unlikely to be the case in Kentish plover chicks because only one of the significant HFCs indicated overdominance whilst the other two were due to underdominance. Another explanation for the lack of heterozygosity and an association with morphological fitness measures is that the genetic component for tarsus and body mass growth is low. However, it has been shown in birds that at least tarsus length is a heritable trait (Alatalo *et al.* 1986, Smith 1993, Larsson 1993). Instead we suggest that fewer genes affect growth than survival and the probability that heterozygosity at one or more of the microsatellite markers were associated with these particular genes is low. Alternatively the allelic composition ('good and bad genes') rather than heterozygosity may have an influence on morphological traits.

***Future directions***

Due to the presumably low linkage disequlibrium in this system the observed MLH fitness correlations provide a unique chance to identify candidate loci that are influential for offspring survival in shorebirds. If HFCs have arisen because of local effects, associated genes should be in the close proximity of the markers, often within a few kilobases (Kruglyak 1999, but see Slatkin 2008). Candidate genes can be identified using genomic maps such as the chicken genome or the shortly expected zebra finch genome. Comparing genotypes at candidate and marker loci would enable us to discriminate between local and direct effect hypothesis. The observed underdominance effects provide a great opportunity to investigate the significance of this pattern and the involved genes may provide further examples of this rarely demonstrated phenomenon.

The magnitude of the genetic effects needs to be studied in different environments to understand whether and how interactions between genes and environments influence

fitness. The use of conserved microsatellites is advantageous for cross population and cross species studies because HFCs can be obtained using the same markers. On the first glance the Tuzla population appears to be an ideal breeding ground for bringing up Kentish plover young. However, competition for resources between families is fierce especially at the end of the breeding season when resources are running low and the magnitude of the genetic effects needs to be compared with the magnitude in other populations/species preferably with lower densities and/or in different geographic regions.

Heterozygosity effects are the most studied and probably the simplest way to associate genes with fitness. In the next step alleles or allele combinations (e.g. 'good' and 'bad' genes) that influence fitness should be identified using stratification tests (Martín-Gálvez *et al.* 2006).

To summarise, we found both positive and negative effects of MLH on a life history trait (offspring survival) but not on morphological traits in a precocial shorebird. The results oppose the view of a positive genomewide effect of heterozygosity, but suggest more complex interactions between heterozygosity and fitness that warrant further investigation.

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**Table 1.** Results of mixed effect Cox regressions for the association between heterozygosity and chick survival. Chick survival was measured as survived days until fledging. Death was the terminal event, disappearance of the entire family or reaching the fledging age of 25 days were considered as censored events in each analysis. Each model contained the heterozygosity estimator and environmental variables as fixed effects (see Methods), but only the results for heterozygosity estimators are presented. Identity of the social or biological family was incorporated into each model as random factor. Full model statistics including the environmental factors are given in the supplementary material.

| | **Random factor: Social family** | | | | | | **Random factor: Biological family** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Heterozygosity marker** | $N_i$, $N_j$ | $s^2_{random}$ | $B \pm SE$ | $e^B$ | $p$ | $q$ | $N_i$, $N_j$ | $s^2_{random}$ | $B \pm SE$ | $e^B$ | $p$ | $q$ |
| *Single locus estimators* | | | | | | | | | | | | |
| Calex-01 | 108, 280 | 0.94 | -0.13 ± 0.38 | 0.88 | 0.73 | 0.12 | 113, 280 | 0.67 | -0.13 ± 0.37 | 0.88 | 0.73 | 0.26 |
| Calex-02 | 108, 280 | 0.94 | 0.09 ± 0.40 | 1.10 | 0.82 | 0.13 | 113, 280 | 0.67 | 0.05 ± 0.39 | 1.06 | 0.89 | 0.29 |
| Calex-04 | 108, 280 | 0.87 | 0.35 ± 0.39 | 1.41 | 0.37 | 0.08 | 113, 280 | 0.60 | 0.34 ± 0.38 | 1.40 | 0.37 | 0.17 |
| Calex-05 | 108, 280 | 1.02 | -0.51 ± 0.31 | 0.60 | 0.10 | 0.04 | 113, 280 | 0.69 | -0.43 ± 0.29 | 0.65 | 0.14 | 0.10 |
| Calex-08 | 108, 280 | 0.95 | -0.26 ± 0.29 | 0.77 | 0.37 | 0.08 | 113, 280 | 0.68 | -0.26 ± 0.28 | 0.77 | 0.36 | 0.17 |
| Calex-12 | 108, 280 | 0.91 | -0.45 ± 0.32 | 0.64 | 0.17 | <0.05 | 113, 280 | 0.66 | -0.44 ± 0.31 | 0.65 | 0.16 | 0.10 |
| Calex-14 | 108, 280 | 0.99 | -0.32 ± 0.46 | 0.73 | 0.49 | 0.09 | 113, 280 | 0.70 | -0.22 ± 0.47 | 0.80 | 0.64 | 0.25 |
| Calex-18 | 108, 280 | 0.89 | -0.63 ± 0.35 | 0.53 | 0.07 | 0.04 | 113, 280 | 0.65 | -0.69 ± 0.34 | 0.50 | 0.04 | 0.06 |
| Calex-19 | 108, 280 | 0.86 | 1.22 ± 0.52 | 3.38 | 0.02 | 0.02 | 113, 280 | 0.58 | 1.21 ± 0.50 | 3.34 | 0.02 | 0.06 |
| Calex-22 | 108, 280 | 0.93 | 0.44 ± 0.30 | 1.56 | 0.14 | <0.05 | 113, 280 | 0.73 | 0.50 ± 0.29 | 1.65 | 0.09 | 0.08 |
| Calex-23 | 108, 280 | 1.19 | 1.14 ± 0.52 | 3.12 | 0.03 | 0.02 | 113, 280 | 0.79 | 0.96 ± 0.49 | 2.60 | 0.05 | 0.06 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Multilocus estimators* | | | | | | | | | | | | |
| Uncorrected heterozygosity | 108, 280 | 0.93 | 0.07 ± 1.17 | 1.07 | 0.95 | Na | 113, 280 | 0.66 | 0.12 ± 1.13 | 1.13 | 0.92 | na |
| Internal relatedness | 108, 280 | 0.93 | -0.08 ± 0.98 | 0.92 | 0.93 | Na | 113, 280 | 0.66 | -0.11 ± 0.95 | 0.89 | 0.91 | na |
| Homozygosity by loci | 108, 280 | 0.93 | -0.09 ± 1.20 | 0.92 | 0.94 | Na | 113, 280 | 0.66 | -0.12 ± 1.16 | 0.89 | 0.92 | na |

$N_i$, number of families; $N_j$, number of chicks; $s^2_{random}$, variance explained by identity; *B,* Cox coefficient; *SE*, standard error; $e^B$, hazard to die before fledging when heterozygosity of the estimator equals 1. A value of 1 means no difference in survival length between completely heterozygous and homozygous individuals, values larger than 1 mean higher risk of deathfor more heterozygous individuals, values lower than 1 mean higher risk of death for more homozygous individuals. *p*, p-value; *q*, q-value, the propability that the p-value is significant due to multiple tests of the same hypothesis. q-values were not calculated for multilocus estimators because they are not independent from each other.

**Table 2.** Results of mixed effects models for the association between heterozygosity and residuals of tarsus growth. Each model contained heterozygosity estimator and environmental variables as fixed effects, but only the results for heterozygosity estimators are presented. Identity of the social or biological family was incorporated into each model as random factor. Full model statistics including the environmental factors are given in the supplementary material.

| | **Random factor: Social family** | | | | | **Random factor: Biological family** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Heterozygosity marker** | $N_i, N_j$ | $s^2_{random}$ | $B \pm SE$ | $p$ | $q$ | $N_i, N_j$ | $s^2_{random}$ | $B \pm SE$ | $p$ | $q$ |
| *Single locus estimators* | | | | | | | | | | |
| Calex-01 | 46, 119 | 0.32 | -0.05 ± 0.23 | 0.84 | 0.88 | 48, 119 | 0.36 | -0.09 ± 0.24 | 0.70 | 0.56 |
| Calex-02 | 46, 119 | 0.32 | 0.07 ± 0.22 | 0.74 | 0.88 | 48, 119 | 0.36 | 0.06 ± 0.22 | 0.78 | 0.56 |
| Calex-04 | 46, 119 | 0.32 | 0.27 ± 0.22 | 0.22 | 0.88 | 48, 119 | 0.35 | 0.20 ± 0.23 | 0.38 | 0.47 |
| Calex-05 | 46, 119 | 0.34 | 0.13 ± 0.20 | 0.53 | 0.88 | 48, 119 | 0.38 | 0.16 ± 0.20 | 0.44 | 0.47 |
| Calex-08 | 46, 119 | 0.33 | -0.11 ± 0.19 | 0.56 | 0.88 | 48, 119 | 0.37 | -0.17 ± 0.19 | 0.38 | 0.47 |
| Calex-12 | 46, 119 | 0.32 | -0.29 ± 0.20 | 0.14 | 0.88 | 48, 119 | 0.35 | -0.19 ± 0.19 | 0.34 | 0.47 |
| Calex-14 | 46, 119 | 0.32 | 0.08 ± 0.26 | 0.77 | 0.88 | 48, 119 | 0.37 | 0.29 ± 0.29 | 0.32 | 0.47 |
| Calex-18 | 46, 119 | 0.33 | -0.07 ± 0.26 | 0.79 | 0.88 | 48, 119 | 0.37 | -0.11 ± 0.25 | 0.68 | 0.56 |
| Calex-19 | 46, 119 | 0.33 | -0.03 ± 0.27 | 0.92 | 0.88 | 48, 119 | 0.37 | 0.01 ± 0.27 | 0.98 | 0.64 |
| Calex-22 | 46, 119 | 0.33 | -0.13 ± 0.16 | 0.44 | 0.88 | 48, 119 | 0.36 | -0.12 ± 0.16 | 0.46 | 0.47 |
| Calex-23 | 46, 119 | 0.34 | -0.24 ± 0.31 | 0.44 | 0.88 | 48, 119 | 0.39 | -0.37 ± 0.31 | 0.23 | 0.47 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Multilocus estimators* | | | | | | | | | | |
| Uncorrected heterozygosity | 46, 119 | 0.33 | -0.34 ± 0.66 | 0.61 | na | 48, 119 | 0.36 | -0.40 ± 0.70 | 0.57 | na |
| Internal relatedness | 46, 119 | 0.33 | 0.38 ± 0.57 | 0.51 | na | 48, 119 | 0.36 | 0.38 ± 0.60 | 0.53 | na |
| Homozygosity by loci | 46, 119 | 0.33 | 0.30 ± 0.67 | 0.65 | na | 48, 119 | 0.36 | 0.36 ± 0.72 | 0.62 | na |

$N_i$, number of families; $N_j$, number of chicks; $s^2_{random}$, variance explained by identity; *B,* slope of the regression line; *SE*, standard error; *p*, p-value; *q*, q-value, the propability that the p-value is significant due to multiple tests of the same hypothesis. q-values were not calculated for multilocus estimators because they are not independent from each other.

**Table 3.** Results of mixed effects models for the association between heterozygosity and residuals of body mass growth. Each model contained heterozygosity estimator and environmental variables as fixed effects, but only the results for heterozygosity estimators are presented. Identity of the social or biological family was incorporated into each model as random factor. Full model statistics including the environmental factors are given in the supplementary material.

| | **Random factor: Social family** | | | | | **Random factor: Biological family** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Heterozygosity marker** | $N_i, N_j$ | $s^2_{random}$ | $B \pm SE$ | $p$ | $Q$ | $N_i, N_j$ | $s^2_{random}$ | $B \pm SE$ | $p$ | $q$ |
| *Single locus estimators* | | | | | | | | | | |
| Calex-01 | 46, 119 | 0.79 | -0.42 ± 0.37 | 0.26 | 0.72 | 48, 119 | 0.61 | -0.54 ± 0.39 | 0.17 | 0.24 |
| Calex-02 | 46, 119 | 0.85 | 0.06 ± 0.36 | 0.86 | 0.95 | 48, 119 | 0.65 | 0.10 ± 0.38 | 0.80 | 0.32 |
| Calex-04 | 46, 119 | 0.83 | 0.16 ± 0.35 | 0.66 | 0.91 | 48, 119 | 0.63 | 0.21 ± 0.38 | 0.59 | 0.32 |
| Calex-05 | 46, 119 | 0.86 | -0.07 ± 0.32 | 0.83 | 0.95 | 48, 119 | 0.67 | -0.12 ± 0.33 | 0.73 | 0.32 |
| Calex-08 | 46, 119 | 0.88 | 0.25 ± 0.31 | 0.42 | 0.77 | 48, 119 | 0.66 | 0.10 ± 0.32 | 0.76 | 0.32 |
| Calex-12 | 46, 119 | 0.85 | -0.67 ± 0.31 | 0.04 | 0.37 | 48, 119 | 0.55 | -0.54 ± 0.33 | 0.11 | 0.23 |
| Calex-14 | 46, 119 | 0.86 | -0.002 ± 0.42 | 1 | 0.37 | 48, 119 | 0.69 | 0.34 ± 0.49 | 0.49 | 0.32 |
| Calex-18 | 46, 119 | 0.87 | 0.03 ± 0.42 | 0.95 | 0.95 | 48, 119 | 0.67 | 0.09 ± 0.42 | 0.83 | 0.32 |
| Calex-19 | 46, 119 | 0.86 | -0.35 ± 0.44 | 0.44 | 0.77 | 48, 119 | 0.64 | -0.35 ± 0.45 | 0.44 | 0.32 |
| Calex-22 | 46, 119 | 0.88 | -0.19 ± 0.27 | 0.49 | 0.77 | 48, 119 | 0.69 | -0.21 ± 0.28 | 0.46 | 0.32 |
| Calex-23 | 46, 119 | 0.94 | -0.90 ± 0.51 | 0.08 | 0.37 | 48, 119 | 0.77 | -1.00 ± 0.51 | 0.05 | 0.21 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Multilocus estimators* | | | | | | | | | | |
| Uncorrected heterozygosity | 46, 119 | 0.90 | -1.42 ± 1.06 | 0.19 | na | 48, 119 | 0.69 | -1.54 ± 1.17 | 0.19 | na |
| Internal relatedness | 46, 119 | 0.90 | 1.33 ± 0.91 | 0.15 | na | 48, 119 | 0.68 | 1.33 ± 1.00 | 0.19 | na |
| Homozygosity by loci | 46, 119 | 0.90 | 1.48 ± 1.09 | 0.18 | na | 48, 119 | 0.69 | 1.55 ± 1.20 | 0.20 | na |

$N_i$, number of families; $N_j$, number of chicks; $s^2_{random}$, variance explained by identity; *B,* slope of the regression line; *SE*, standard error; *p*, p-value; *q*, q-value, the propability that the p-value is significant due to multiple tests of the same hypothesis. q-values were not calculated for multilocus estimators because they are not independent from each other.

# Supplementary Material

## Cox Mixed Effects Models

Abbreviations:

$n_i$ - number of families

$n_j$ - number of chicks

exp(B) - hazard of death. Values from 0 to 1 indicate decrease of death hazard, values larger than 1 indicate that death hazard increased.

### Survival in days until fledging – Random factor: Social family

#### Environmental variables only

$n_i/n_j$=108/280
Iterations= 3 54

```
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 368.4941 - 344.8289

Penalized loglik: chisq= 79.45 on 38.31 degrees of freedom, p= 0.00011
Integrated loglik: chisq= 32.12 on 5 degrees of freedom, p= 5.6e-06
```

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.12 | 0.89 | 0.27 | - 0.44 | 0.66 |
| Site quality | 0.25 | 1.28 | 0.39 | 0.63 | 0.53 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.92 | 0.36 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.13 | <0.001 |

Random effect variance: 0.937

#### Calex-01

$n_i/n_j$=108/280
Iterations= 3 54

```
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 368.4389 - 344.7018

Penalized loglik: chisq= 79.7 on 39.23 degrees of freedom, p= 0.00014
Integrated loglik: chisq= 32.23 on 6 degrees of freedom, p= 1.5e-05
```

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.11 | 0.90 | 0.27 | - 0.40 | 0.69 |
| Site quality | 0.25 | 1.28 | 0.39 | 0.63 | 0.53 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.92 | 0.36 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.14 | <0.001 |
| Ho Calex-01 | - 0.13 | 0.88 | 0.38 | - 0.34 | 0.73 |

Random effect variance: 0.941

#### Calex-02

$n_i/n_j$=108/280
Iterations= 3 54

```
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 368.4679 - 344.7742

Penalized loglik: chisq= 79.56 on 39.18 degrees of freedom, p= 0.00015
Integrated loglik: chisq= 32.17 on 6 degrees of freedom, p= 1.5e-05
```

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.12 | 0.89 | 0.27 | - 0.45 | 0.65 |
| Site quality | 0.25 | 1.29 | 0.39 | 0.64 | 0.52 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.90 | 0.37 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.08 | <0.001 |
| Ho Calex-02 | 0.09 | 1.10 | 0.40 | 0.23 | 0.82 |

Random effect variance: 0.939

## Calex-04

$n_i/n_j$=108/280
  Iterations= 3 54

```
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 368.0666 - 345.6114

Penalized loglik: chisq= 77.88 on 37.85 degrees of freedom, p= 0.00014
Integrated loglik: chisq= 32.97 on 6 degrees of freedom, p= 1.1e-05
```

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.11 | 0.90 | 0.27 | - 0.41 | 0.68 |
| Site quality | 0.30 | 1.34 | 0.39 | 0.76 | 0.45 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.92 | 0.36 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.18 | <0.001 |
| Ho Calex-04 | 0.35 | 1.41 | 0.39 | 0.90 | 0.37 |

```
Random effect variance: 0.872
```

## Calex-05

$n_i/n_j$=108/280
  Iterations= 3 46

```
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 367.0763 - 342.0721

Penalized loglik: chisq= 84.96 on 40.4 degrees of freedom, p= 5.2e-05
Integrated loglik: chisq= 34.95 on 6 degrees of freedom, p= 4.4e-06
```

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.09 | 0.91 | 0.27 | - 0.34 | 0.73 |
| Site quality | 0.21 | 1.24 | 0.40 | 0.52 | 0.60 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.01 | 0.31 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.09 | <0.001 |
| Ho Calex-05 | - 0.51 | 0.60 | 0.31 | - 1.66 | 0.10 |

```
Random effect variance: 1.017
```

## Calex-08

$n_i/n_j$=108/280
  Iterations= 3 54

```
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 368.1295  - 344.223

Penalized loglik: chisq= 80.66 on 39.44 degrees of freedom, p= 0.00012
Integrated loglik: chisq= 32.85 on 6 degrees of freedom, p= 1.1e-05
```

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.11 | 0.90 | 0.27 | - 0.41 | 0.68 |
| Site quality | 0.22 | 1.25 | 0.39 | 0.56 | 0.58 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.97 | 0.33 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.15 | <0.001 |
| Ho Calex-08 | - 0.26 | 0.77 | 0.29 | - 0.90 | 0.37 |

```
Random effect variance: 0.949
```

## Calex-12

$n_i/n_j$=108/280
  Iterations= 3 54

```
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 367.5795 - 344.4852

Penalized loglik: chisq= 80.13 on 38.5 degrees of freedom, p= 9.5e-05
Integrated loglik: chisq= 33.95 on 6 degrees of freedom, p= 6.9e-06
```

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.11 | 0.90 | 0.27 | - 0.41 | 0.68 |
| Site quality | 0.31 | 1.37 | 0.39 | 0.79 | 0.43 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.07 | 0.28 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.19 | <0.001 |
| Ho Calex-12 | - 0.45 | 0.64 | 0.32 | - 1.37 | 0.17 |

```
Random effect variance: 0.906
```

## Calex-14

$n_i/n_j$=108/280
  Iterations= 3 54

| | NULL | Integrated | Penalized |
|---|---|---|---|
| Log-likelihood | - 384.5525 | - 368.2745 | - 343.7074 |

Penalized loglik: chisq= 81.69 on 40.13 degrees of freedom, p= 0.00012
Integrated loglik: chisq= 32.56 on 6 degrees of freedom, p= 1.3e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.12 | 0.89 | 0.27 | - 0.44 | 0.66 |
| Site quality | 0.24 | 1.28 | 0.40 | 0.61 | 0.54 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.82 | 0.41 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.09 | <0.001 |
| Ho Calex-14 | - 0.32 | 0.73 | 0.46 | - 0.70 | 0.49 |

Random effect variance: 0.987

## Calex-18

$n_i/n_j$=108/280
  Iterations= 3 54

| | NULL | Integrated | Penalized |
|---|---|---|---|
| Log-likelihood | - 384.5525 | - 366.853 | - 344.2342 |

Penalized loglik: chisq= 80.64 on 37.8 degrees of freedom, p= 6.2e-05
Integrated loglik: chisq= 35.4 on 6 degrees of freedom, p= 3.6e-06

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.05 | 0.95 | 0.27 | - 0.19 | 0.85 |
| Site quality | 0.31 | 1.36 | 0.39 | 0.79 | 0.43 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.89 | 0.37 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.22 | <0.001 |
| Ho Calex-18 | - 0.63 | 0.53 | 0.35 | - 1.80 | 0.072 |

Random effect variance: 0.886

## Calex-19

$n_i/n_j$=108/280
  Iterations= 3 54

| | NULL | Integrated | Penalized |
|---|---|---|---|
| Log-likelihood | - 384.5525 | - 364.8658 | - 342.8708 |

Penalized loglik: chisq= 83.36 on 37.07 degrees of freedom, p= 2e-05
Integrated loglik: chisq= 39.37 on 6 degrees of freedom, p= 6e-07

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.12 | 0.89 | 0.27 | - 0.44 | 0.66 |
| Site quality | 0.34 | 1.40 | 0.39 | 0.86 | 0.39 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.55 | 0.58 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.26 | <0.001 |
| Ho Calex-19 | 1.22 | 3.38 | 0.52 | 2.35 | 0.019 |

Random effect variance: 0.858

## Calex-22

$n_i/n_j$=108/280
  Iterations= 3 54

| | NULL | Integrated | Penalized |
|---|---|---|---|
| Log-likelihood | - 384.5525 | - 367.2625 | - 343.9 |

Penalized loglik: chisq= 81.31 on 38.73 degrees of freedom, p= 7.5e-05
Integrated loglik: chisq= 34.58 on 6 degrees of freedom, p= 5.2e-06

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.14 | 0.87 | 0.27 | - 0.52 | 0.61 |
| Site quality | 0.22 | 1.26 | 0.39 | 0.56 | 0.58 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.92 | 0.36 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.08 | <0.001 |
| Ho Calex-22 | 0.44 | 1.56 | 0.30 | 1.49 | 0.14 |

Random effect variance: 0.925

## Calex-23

$n_i/n_j$=108/280
  Iterations= 3 38

| | NULL | Integrated | Penalized |
|---|---|---|---|
| Log-likelihood | - 384.5525 | - 365.6877 | - 337.8789 |

Penalized loglik: chisq= 93.35 on 43.3 degrees of freedom, p= 1.6e-05
Integrated loglik: chisq= 37.73 on 6 degrees of freedom, p= 1.3e-06

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.15 | 0.86 | 0.27 | - 0.57 | 0.57 |
| Site quality | 0.27 | 1.31 | 0.42 | 0.65 | 0.52 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.60 | 0.55 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.00 | <0.001 |
| Ho Calex-23 | 1.14 | 3.12 | 0.52 | 2.19 | 0.029 |

Random effect variance: 1.185

## Uncorrected heterozygosity across all loci

$n_i/n_j$=108/280
  Iterations= 3 54

| | NULL | Integrated | Penalized |
|---|---|---|---|
| Log-likelihood | - 384.5525 | - 368.4906 | - 344.8919 |

Penalized loglik: chisq= 79.32 on 39.07 degrees of freedom, p= 0.00015
Integrated loglik: chisq= 32.12 on 6 degrees of freedom, p= 1.5e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.12 | 0.89 | 0.27 | - 0.44 | 0.66 |
| Site quality | 0.25 | 1.28 | 0.39 | 0.63 | 0.53 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.90 | 0.37 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.11 | <0.001 |
| Ho ALL | 0.07 | 1.07 | 1.17 | 0.06 | 0.95 |

Random effect variance: 0.933

## 'Homozygosity by loci' across all loci

$n_i/n_j$=108/280
  Iterations= 3 54

| | NULL | Integrated | Penalized |
|---|---|---|---|
| Log-likelihood | - 384.5525 | - 368.4895 | - 344.9006 |

Penalized loglik: chisq= 79.3 on 39.06 degrees of freedom, p= 0.00015
Integrated loglik: chisq= 32.13 on 6 degrees of freedom, p= 1.5e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.12 | 0.89 | 0.27 | - 0.44 | 0.66 |
| Site quality | 0.25 | 1.28 | 0.39 | 0.63 | 0.53 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.89 | 0.37 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.11 | <0.001 |
| HL ALL | - 0.09 | 0.92 | 1.20 | - 0.07 | 0.94 |

Random effect variance: 0.933

## 'Internal relatedness' across all loci

$n_i/n_j$=108/280
  Iterations= 3 54

| | NULL | Integrated | Penalized |
|---|---|---|---|
| Log-likelihood | - 384.5525 | - 368.4884 | - 344.8965 |

Penalized loglik: chisq= 79.31 on 39.07 degrees of freedom, p= 0.00015
Integrated loglik: chisq= 32.13 on 6 degrees of freedom, p= 1.5e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.12 | 0.89 | 0.27 | - 0.44 | 0.66 |
| Site quality | 0.25 | 1.28 | 0.39 | 0.63 | 0.53 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.90 | 0.37 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.11 | <0.001 |
| IR ALL | - 0.08 | 0.92 | 0.98 | - 0.08 | 0.93 |

Random effect variance: 0.933

# Survival in days until fledging – Random factor: Biological family

## Environmental variables only

$n_i/n_j$=113/280
  Iterations= 3 62
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 369.9672 - 351.3245

Penalized loglik: chisq= 66.46 on 32.95 degrees of freedom, p= 0.00048
Integrated loglik: chisq= 29.17 on 5 degrees of freedom, p= 2.1e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.07 | 0.93 | 0.26 | - 0.29 | 0.77 |
| Site quality | 0.18 | 1.20 | 0.36 | 0.51 | 0.61 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.10 | 0.27 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.28 | <0.001 |

Random effect variance: 0.668

## Calex-01

$n_i/n_j$=113/280
  Iterations= 3 62
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 369.9092 - 351.1769

Penalized loglik: chisq= 66.75 on 33.91 degrees of freedom, p= 0.00064
Integrated loglik: chisq= 29.29 on 6 degrees of freedom, p= 5.4e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.07 | 0.93 | 0.26 | - 0.26 | 0.79 |
| Site quality | 0.19 | 1.21 | 0.36 | 0.52 | 0.60 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.10 | 0.27 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.29 | <0.001 |
| Ho Calex-01 | - 0.13 | 0.88 | 0.37 | - 0.35 | 0.73 |

Random effect variance: 0.672

## Calex-02

$n_i/n_j$=113/280
  Iterations= 3 62
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 369.9566 - 351.3249

Penalized loglik: chisq= 66.46 on 33.77 degrees of freedom, p= 0.00066
Integrated loglik: chisq= 29.19 on 6 degrees of freedom, p= 5.6e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.08 | 0.93 | 0.26 | - 0.29 | 0.77 |
| Site quality | 0.19 | 1.21 | 0.36 | 0.52 | 0.60 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.08 | 0.28 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.23 | <0.001 |
| Ho Calex-02 | 0.05 | 1.06 | 0.39 | 0.14 | 0.89 |

Random effect variance: 0.668

## Calex-04

$n_i/n_j$=113/280
  Iterations= 3 46
                    NULL Integrated Penalized
Log-likelihood - 384.5525   - 369.546 - 352.2688

Penalized loglik: chisq= 64.57 on 32.07 degrees of freedom, p= 0.00058
Integrated loglik: chisq= 30.01 on 6 degrees of freedom, p= 3.9e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.08 | 0.93 | 0.26 | - 0.29 | 0.77 |
| Site quality | 0.24 | 1.27 | 0.36 | 0.66 | 0.51 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.10 | 0.27 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.34 | <0.001 |
| Ho Calex-04 | 0.34 | 1.40 | 0.38 | 0.89 | 0.37 |

Random effect variance: 0.601

## Calex-05

$n_i/n_j$=113/280
  Iterations= 3 62
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 368.8303 - 349.6875

Penalized loglik: chisq= 69.73 on 34.28 degrees of freedom, p= 0.00033
Integrated loglik: chisq= 31.44 on 6 degrees of freedom, p= 2.1e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.05 | 0.95 | 0.26 | - 0.19 | 0.85 |
| Site quality | 0.15 | 1.16 | 0.37 | 0.41 | 0.68 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.19 | 0.24 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.26 | <0.001 |
| Ho Calex-05 | - 0.43 | 0.65 | 0.29 | - 1.49 | 0.14 |

Random effect variance: 0.694

## Calex-08

$n_i/n_j$=113/280
  Iterations= 3 62
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 369.5577  - 350.569

Penalized loglik: chisq= 67.97 on 34.23 degrees of freedom, p= 0.00052
Integrated loglik: chisq= 29.99 on 6 degrees of freedom, p= 3.9e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.06 | 0.94 | 0.26 | - 0.24 | 0.81 |
| Site quality | 0.15 | 1.16 | 0.36 | 0.41 | 0.68 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.15 | 0.25 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.30 | <0.001 |
| Ho Calex-08 | - 0.26 | 0.77 | 0.28 | - 0.92 | 0.36 |

Random effect variance: 0.684

## Calex-12

$n_i/n_j$=113/280
  Iterations= 3 70
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 369.0234  - 350.648

Penalized loglik: chisq= 67.81 on 33.45 degrees of freedom, p= 4e-04
Integrated loglik: chisq= 31.06 on 6 degrees of freedom, p= 2.5e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.07 | 0.93 | 0.26 | - 0.28 | 0.78 |
| Site quality | 0.25 | 1.28 | 0.36 | 0.68 | 0.50 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.25 | 0.21 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.35 | <0.001 |
| Ho Calex-12 | - 0.44 | 0.65 | 0.31 | - 1.40 | 0.16 |

Random effect variance: 0.657

## Calex-14

$n_i/n_j$=113/280
  Iterations= 3 62
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 369.8639 - 350.6188

Penalized loglik: chisq= 67.87 on 34.47 degrees of freedom, p= 0.00059
Integrated loglik: chisq= 29.38 on 6 degrees of freedom, p= 5.2e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.08 | 0.93 | 0.26 | - 0.29 | 0.77 |
| Site quality | 0.18 | 1.20 | 0.36 | 0.50 | 0.62 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.02 | 0.31 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.26 | <0.001 |
| Ho Calex-14 | - 0.22 | 0.80 | 0.47 | - 0.46 | 0.64 |

Random effects variance: 0.696

## Calex-18

$n_i/n_j$=113/280
  Iterations= 3 62
                    NULL Integrated Penalized
Log-likelihood - 384.5525    - 367.93 - 349.6621

  Penalized loglik: chisq= 69.78 on 33.16 degrees of freedom, p= 0.00021
 Integrated loglik: chisq= 33.24 on 6 degrees of freedom, p= 9.4e-06

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.01 | 1.00 | 0.26 | - 0.02 | 0.99 |
| Site quality | 0.26 | 1.30 | 0.36 | 0.72 | 0.47 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.03 | 0.30 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.34 | <0.001 |
| Ho Calex-18 | - 0.69 | 0.50 | 0.34 | - 2.05 | 0.04 |

Random effect variance: 0.654

## Calex-19

$n_i/n_j$=113/280
  Iterations= 3 46
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 366.2252 - 349.7319

  Penalized loglik: chisq= 69.64 on 30.88 degrees of freedom, p= 8.1e-05
 Integrated loglik: chisq= 36.65 on 6 degrees of freedom, p= 2.1e-06

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.08 | 0.92 | 0.26 | - 0.32 | 0.75 |
| Site quality | 0.27 | 1.31 | 0.36 | 0.76 | 0.45 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.72 | 0.47 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.44 | <0.001 |
| Ho Calex-19 | 1.21 | 3.34 | 0.50 | 2.39 | 0.017 |

Random effect variance: 0.580

## Calex-22

$n_i/n_j$=113/280
  Iterations= 3 62
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 368.3482 - 348.3818

Penalized loglik: chisq= 72.34 on 35.31 degrees of freedom, p= 0.00024
Integrated loglik: chisq= 32.41 on 6 degrees of freedom, p= 1.4e-05

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.10 | 0.91 | 0.26 | - 0.37 | 0.71 |
| Site quality | 0.15 | 1.16 | 0.37 | 0.40 | 0.69 |
| Hatch date | 0.01 | 1.01 | 0.01 | 1.08 | 0.28 |
| Length of BP care | - 0.08 | 0.92 | 0.02 | - 4.19 | <0.001 |
| Ho Calex-22 | 0.50 | 1.65 | 0.29 | 1.72 | 0.085 |

Random effect variance: 0.733

## Calex-23

$n_i/n_j$=113/280
  Iterations= 3 62
                    NULL Integrated Penalized
Log-likelihood - 384.5525  - 367.7045 - 346.5054

Penalized loglik: chisq= 76.09 on 36.79 degrees of freedom, p= 0.00015
Integrated loglik: chisq= 33.7 on 6 degrees of freedom, p= 7.7e-06

| | B | exp(B) | SE(B) | z | p |
|---|---|---|---|---|---|
| Sex | - 0.10 | 0.90 | 0.26 | - 0.40 | 0.69 |
| Site quality | 0.18 | 1.20 | 0.38 | 0.49 | 0.62 |
| Hatch date | 0.01 | 1.01 | 0.01 | 0.80 | 0.42 |
| Length of BP care | - 0.08 | 0.92 | 0.01 | - 4.23 | <0.001 |
| Ho Calex-23 | 0.96 | 2.60 | 0.49 | 1.94 | 0.052 |

Random effects variance: 0.792

## Uncorrected heterozygosity across all loci

```
ni/nj=113/280
  Iterations= 3 62
                   NULL Integrated Penalized
Log-likelihood - 384.5525  - 369.9588 - 351.4036

Penalized loglik: chisq= 66.3 on 33.67 degrees of freedom, p= 0.00067
Integrated loglik: chisq= 29.19 on 6 degrees of freedom, p= 5.6e-05

                      B             exp(B)         SE(B)     z           p
Sex                 - 0.08          0.92           0.26    - 0.30        0.77
Site quality          0.19          1.21           0.36      0.52        0.60
Hatch date            0.01          1.01           0.01      1.07        0.29
Length of BP care   - 0.08          0.92           0.02    - 4.26        <0.001
Ho ALL                0.12          1.13           1.13      0.11        0.92

Random effect variance: 0.664
```

## 'Homozygosity by loci' across all loci

```
ni/nj=113/280
  Iterations= 3 62
                   NULL Integrated Penalized
Log-likelihood - 384.5525  - 369.9596 - 351.4135

  Penalized loglik: chisq= 66.28 on 33.66 degrees of freedom, p= 0.00067
 Integrated loglik: chisq= 29.19 on 6 degrees of freedom, p= 5.6e-05

                      B             exp(B)         SE(B)     z           p
Sex                 - 0.08          0.92           0.26    - 0.30        0.77
Site quality          0.19          1.21           0.36      0.52        0.60
Hatch date            0.01          1.01           0.01      1.07        0.29
Length of BP care   - 0.08          0.92           0.02    - 4.26        <0.001
HL ALL              - 0.12          0.89           1.16    - 0.10        0.92

Random effects variance: 0.664
```

## 'Internal relatedness' across all loci

```
ni/nj=113/280
  Iterations= 3 62
                   NULL Integrated Penalized
Log-likelihood - 384.5525  - 369.9574 - 351.3942

  Penalized loglik: chisq= 66.32 on 33.69 degrees of freedom, p= 0.00067
 Integrated loglik: chisq= 29.19 on 6 degrees of freedom, p= 5.6e-05

                      B             exp(B)         SE(B)     z           p
Sex                 - 0.08          0.92           0.26    - 0.30        0.77
Site quality          0.19          1.21           0.36      0.52        0.60
Hatch date            0.01          1.01           0.01      1.07        0.29
Length of BP care   - 0.08          0.92           0.02    - 4.27        <0.001
IR ALL              - 0.11          0.89           0.95    - 0.12        0.91

Random effect variance: 0.665
```

# Linear mixed effects models

Abbreviations:

AIC – Akaike information criteria

BIC – Bayesian information criteria

LogLik - log likelihood

StdDev - standard deviation

## Mean residuals of tarsus growth – Random factor: Social family

### Environmental variables only

```
       AIC      BIC    logLik
  337.6978 356.8511 - 161.8489

Random effects:

         (Intercept)  Residual
StdDev:    0.569806 0.7507872

                      B             SE(B)   DF      t             p
(Intercept)           - 0.87        0.79    71      - 1.09        0.28
Sex                     0.32        0.16    71        2.05        0.04
Site quality          - 0.80        0.38    43      - 2.12        0.04
Hatch date              0.01        0.01    71        1.05        0.30
Length of BP care       0           0.01    43      - 0.05        0.96

Number of Observations: 119
Number of Groups: 46
```

### Calex-01

```
       AIC      BIC    logLik
  340.7654 362.5845 - 162.3827

Random effects:

        (Intercept)  Residual
StdDev:   0.5682187 0.7553413

                      B             SE(B)   DF      t             p
(Intercept)           - 0.82        0.81    70      - 1.02        0.31
Sex                     0.33        0.16    70        2.05        0.04
Site quality          - 0.80        0.38    43      - 2.12        0.04
Hatch date              0.01        0.01    70        1.05        0.30
Length of BP care       0           0.01    43      - 0.04        0.97
Ho Calex-01           - 0.05        0.23    70      - 0.20        0.84

Number of Observations: 119
Number of Groups: 46
```

### Calex-02

```
       AIC      BIC    logLik
  340.7797 362.5988 - 162.3899

Random effects:

        (Intercept)  Residual
StdDev:   0.5620682 0.7571663

                      B             SE(B)   DF      t             p
(Intercept)           - 0.87        0.78    70      - 1.11        0.27
Sex                     0.31        0.16    70        1.97        0.05
Site quality          - 0.80        0.38    43      - 2.12        0.04
Hatch date              0.01        0.01    70        0.97        0.34
Length of BP care       0           0.01    43      - 0.01        0.99
Ho Calex-02             0.07        0.22    70        0.33        0.74

Number of Observations: 119
Number of Groups: 46
```

## Calex-04

```
      AIC      BIC    logLik
  339.399 361.2181 - 161.6995

Random effects:

        (Intercept) Residual
StdDev:   0.5619637 0.751272

                      B             SE(B)   DF      t             p
(Intercept)           - 0.98        0.79    70      - 1.25        0.22
Sex                     0.31        0.16    70        1.98        0.05
Site quality          - 0.79        0.38    43      - 2.11        0.04
Hatch date              0.01        0.01    70        0.95        0.34
Length of BP care       0           0.01    43      - 0.11        0.92
Ho Calex-04             0.27        0.22    70        1.24        0.22

Number of Observations: 119
Number of Groups: 46
```

## Calex-05

```
      AIC      BIC    logLik
  340.695 362.5141 - 162.3475

Random effects:

        (Intercept)  Residual
StdDev:   0.5796653 0.7499253

                      B             SE(B)   DF      t             p
(Intercept)           - 0.88        0.80    70      - 1.10        0.27
Sex                     0.31        0.16    70        1.96        0.05
Site quality          - 0.80        0.38    43      - 2.08        0.04
Hatch date              0.01        0.01    70        0.97        0.34
Length of BP care       0           0.01    43      - 0.11        0.91
Ho Calex-05             0.13        0.20    70        0.64        0.53

Number of Observations: 119
Number of Groups: 46
```

## Calex-08

```
       AIC      BIC    logLik
  340.8201 362.6392 - 162.4101

Random effects:

        (Intercept)  Residual
StdDev:   0.5759824 0.7513963

                      B             SE(B)   DF      t             p
(Intercept)           - 0.82        0.79    70      - 1.04        0.30
Sex                     0.32        0.16    70        2.07        0.04
Site quality          - 0.81        0.38    43      - 2.13        0.04
Hatch date              0.01        0.01    70        1.10        0.28
Length of BP care       0           0.01    43      - 0.10        0.93
Ho Calex-08           - 0.11        0.19    70      - 0.59        0.56

Number of Observations: 119
Number of Groups: 46
```

## Calex-12

```
      AIC      BIC    logLik
  338.947 360.7661 - 161.4735

Random effects:

        (Intercept) Residual
StdDev:   0.5684457   0.7464

                      B             SE(B)   DF      t             p
(Intercept)           - 0.65        0.80    70      - 0.81        0.42
Sex                     0.31        0.16    70        1.98        0.05
Site quality          - 0.85        0.38    43      - 2.24        0.03
Hatch date              0.01        0.01    70        1.08        0.28
Length of BP care       0           0.01    43        0           1
Ho Calex-12           - 0.29        0.20    70      - 1.48        0.14

Number of Observations: 119
Number of Groups: 46
```

## Calex-14

```
      AIC      BIC    logLik
  340.4796 362.2987 - 162.2398

Random effects:

        (Intercept)  Residual
StdDev:   0.5662378 0.7558222

                      B             SE(B)   DF      t             p
(Intercept)           - 0.97        0.88    70      - 1.11        0.27
Sex                     0.33        0.16    70        2.06        0.04
Site quality          - 0.82        0.38    43      - 2.14        0.04
Hatch date              0.01        0.01    70        1.09        0.28
Length of BP care       0           0.01    43      - 0.06        0.95
Ho Calex-14             0.08        0.26    70        0.30        0.77

Number of Observations: 119
Number of Groups: 46
```

## Calex-18

```
      AIC      BIC    logLik
  340.5248 362.3439 - 162.2624

Random effects:

        (Intercept)  Residual
StdDev:   0.5743649 0.7531181

                      B             SE(B)   DF      t             p
(Intercept)           - 0.82        0.80    70      - 1.02        0.31
Sex                     0.32        0.16    70        2.05        0.04
Site quality          - 0.80        0.38    43      - 2.10        0.04
Hatch date              0.01        0.01    70        1.06        0.29
Length of BP care       0           0.01    43      - 0.06        0.96
Ho Calex-18           - 0.07        0.26    70      - 0.26        0.79

Number of Observations: 119
Number of Groups: 46
```

## Calex-19

```
       AIC      BIC    logLik
  340.4465 362.2656 - 162.2232

Random effects:

        (Intercept)  Residual
StdDev:   0.5717105 0.7542684

                      B             SE(B)   DF      t             p
(Intercept)           - 0.82        0.87    70      - 0.95        0.35
Sex                     0.32        0.16    70        2.00        0.05
Site quality          - 0.80        0.38    43      - 2.11        0.04
Hatch date              0.01        0.01    70        1.02        0.31
Length of BP care       0           0.01    43      - 0.04        0.96
Ho Calex-19           - 0.03        0.27    70      - 0.10        0.92

Number of Observations: 119
Number of Groups: 46
```

## Calex-22

```
       AIC      BIC    logLik
  340.8682 362.6873 - 162.4341

Random effects:

        (Intercept)  Residual
StdDev:   0.5720618 0.7516728

                      B             SE(B)   DF      t             p
(Intercept)           - 0.76        0.80    70      - 0.94        0.35
Sex                     0.32        0.16    70        2.04        0.04
Site quality          - 0.78        0.38    43      - 2.03        0.05
Hatch date              0.01        0.01    70        1.00        0.32
Length of BP care       0           0.01    43      - 0.01        0.99
Ho Calex-22           - 0.13        0.16    70      - 0.78        0.44

Number of Observations: 119
Number of Groups: 46
```

## Calex-23

```
       AIC      BIC    logLik
  339.5935 361.4126 - 161.7967

Random effects:

        (Intercept)  Residual
StdDev:   0.5817861 0.7484416

                      B             SE(B)   DF      t             p
(Intercept)           - 0.64        0.84    70      - 0.75        0.45
Sex                     0.32        0.16    70        2.03        0.05
Site quality          - 0.78        0.38    43      - 2.03        0.05
Hatch date              0.01        0.01    70        1.04        0.30
Length of BP care       0           0.01    43      - 0.06        0.95
Ho Calex-23           - 0.24        0.31    70      - 0.77        0.44

Number of Observations: 119
Number of Groups: 46
```

## Uncorrected heterozygosity across all loci

```
       AIC     BIC    logLik
  338.4329 360.252 - 161.2164

Random effects:

        (Intercept)  Residual
StdDev:   0.5738552 0.7524996

                      B             SE(B)   DF      t             p
(Intercept)           - 0.63        0.91    70      - 0.69        0.49
Sex                     0.32        0.16    70        2.06        0.04
Site quality          - 0.80        0.38    43      - 2.10        0.04
Hatch date              0.01        0.01    70        1.08        0.28
Length of BP care       0           0.01    43      - 0.04        0.97
Ho ALL                - 0.34        0.66    70      - 0.51        0.61

Number of Observations: 119
Number of Groups: 46
```

## 'Internal relatedness' across all loci

```
       AIC      BIC    logLik
  338.5594 360.3785 - 161.2797

Random effects:

        (Intercept)  Residual
StdDev:   0.5731682 0.7519707

                      B             SE(B)   DF      t             p
(Intercept)           - 0.91        0.79    70      - 1.14        0.26
Sex                     0.32        0.16    70        2.06        0.04
Site quality          - 0.80        0.38    43      - 2.11        0.04
Hatch date              0.01        0.01    70        1.10        0.28
Length of BP care       0           0.01    43      - 0.03        0.98
IR ALL                  0.38        0.57    70        0.66        0.51

Number of Observations: 119
Number of Groups: 46
```

## 'Homozygosity by loci' across all loci

```
       AIC     BIC    logLik
  338.4479 360.267 - 161.2240

Random effects:

        (Intercept)  Residual
StdDev:   0.5738056 0.7527462

                      B             SE(B)   DF      t             p
(Intercept)           - 0.95        0.82    70      - 1.16        0.25
Sex                     0.32        0.16    70        2.06        0.04
Site quality          - 0.80        0.38    43      - 2.10        0.04
Hatch date              0.01        0.01    70        1.08        0.29
Length of BP care       0           0.01    43      - 0.04        0.97
HL ALL                  0.30        0.67    70        0.45        0.65

Number of Observations: 119
Number of Groups: 46
```

# Mean residuals of tarsus growth – Random factor: Biological family

## Environmental variables only

```
       AIC     BIC    logLik
  336.2236 355.377 - 161.1118

Random effects:

        (Intercept)  Residual
StdDev:    0.603275 0.7234626

                       B             SE(B)   DF      t             p
(Intercept)          - 0.72          0.79    67      - 0.91        0.36
Sex                    0.27          0.16    67        1.72        0.09
Site quality         - 0.65          0.33    67      - 1.97        0.05
Hatch date             0.01          0.01    67        1           0.32
Length of BP care    - 0.01          0.01    67      - 0.73        0.47

Number of Observations: 119
Number of Groups: 48
```

## Calex-01

```
       AIC      BIC    logLik
  339.0961 360.9152 - 161.5481

Random effects:

        (Intercept)  Residual
StdDev:   0.6008635 0.7279143

                       B             SE(B)   DF      t             p
(Intercept)          - 0.66          0.81    66      - 0.82        0.42
Sex                    0.28          0.16    66        1.76        0.08
Site quality         - 0.65          0.33    66      - 1.97        0.05
Hatch date             0.01          0.01    66        1.01        0.32
Length of BP care    - 0.01          0.01    66      - 0.69        0.50
Ho Calex-01          - 0.09          0.24    66      - 0.39        0.70

Number of Observations: 119
Number of Groups: 48
```

## Calex-02

```
       AIC      BIC    logLik
  339.3147 361.1338 - 161.6573

Random effects:

        (Intercept)  Residual
StdDev:   0.5979011 0.7293453

                       B             SE(B)   DF      t             p
(Intercept)          - 0.73          0.79    66      - 0.93        0.36
Sex                    0.26          0.16    66        1.64        0.10
Site quality         - 0.64          0.33    66      - 1.96        0.05
Hatch date             0.01          0.01    66        0.91        0.36
Length of BP care    - 0.01          0.01    66      - 0.66        0.51
Ho Calex-02            0.06          0.22    66        0.29        0.78

Number of Observations: 119
Number of Groups: 48
```

## Calex-04

```
       AIC      BIC    logLik
  338.6068 360.4259 - 161.3034

Random effects:

        (Intercept)  Residual
StdDev:   0.5886003 0.7299661

                      B             SE(B)   DF      t             p
(Intercept)           - 0.83        0.79    66      - 1.05        0.30
Sex                     0.27        0.16    66        1.70        0.09
Site quality          - 0.64        0.33    66      - 1.95        0.06
Hatch date              0.01        0.01    66        0.94        0.35
Length of BP care     - 0.01        0.01    66      - 0.75        0.46
Ho Calex-04             0.20        0.23    66        0.88        0.38

Number of Observations: 119
Number of Groups: 48
```

## Calex-05

```
       AIC      BIC    logLik
  339.0050 360.8241 - 161.5025

Random effects:

        (Intercept)  Residual
StdDev:   0.6200276 0.7189261

                      B             SE(B)   DF      t             p
(Intercept)           - 0.73        0.80    66      - 0.91        0.37
Sex                     0.25        0.16    66        1.58        0.12
Site quality          - 0.64        0.33    66      - 1.93        0.06
Hatch date              0.01        0.01    66        0.88        0.38
Length of BP care     - 0.01        0.01    66      - 0.81        0.41
Ho Calex-05             0.16        0.20    66        0.78        0.44

Number of Observations: 119
Number of Groups: 48
```

## Calex-08

```
       AIC      BIC    logLik
  338.9448 360.7639 - 161.4724

Random effects:

        (Intercept)  Residual
StdDev:   0.6136171 0.7205364

                      B             SE(B)   DF      t             p
(Intercept)           - 0.65        0.80    66      - 0.81        0.42
Sex                     0.27        0.16    66        1.75        0.09
Site quality          - 0.64        0.33    66      - 1.92        0.06
Hatch date              0.01        0.01    66        1.05        0.30
Length of BP care     - 0.01        0.01    66      - 0.74        0.46
Ho Calex-08           - 0.17        0.19    66      - 0.88        0.38

Number of Observations: 119
Number of Groups: 48
```

## Calex-12

```
       AIC      BIC    logLik
  338.7572 360.5763 - 161.3786

Random effects:

        (Intercept)  Residual
StdDev:   0.5884513 0.7294068

                      B             SE(B)   DF      t             p
(Intercept)           - 0.60        0.79    66      - 0.76        0.45
Sex                     0.26        0.16    66        1.69        0.10
Site quality          - 0.68        0.33    66      - 2.08        0.04
Hatch date              0.01        0.01    66        1.05        0.30
Length of BP care     - 0.01        0.01    66      - 0.74        0.46
Ho Calex-12           - 0.19        0.19    66      - 0.96        0.34

Number of Observations: 119
Number of Groups: 48
```

## Calex-14

```
       AIC      BIC    logLik
  337.8658 359.6849 - 160.9329

Random effects:

        (Intercept)  Residual
StdDev:   0.6122577 0.7201988

                      B             SE(B)   DF      t             p
(Intercept)           - 1.16        0.91    66      - 1.27        0.21
Sex                     0.30        0.16    66        1.87        0.07
Site quality          - 0.69        0.33    66      - 2.07        0.04
Hatch date              0.01        0.01    66        1.18        0.24
Length of BP care     - 0.01        0.01    66      - 0.76        0.45
Ho Calex-14             0.29        0.29    66        0.99        0.32

Number of Observations: 119
Number of Groups: 48
```

## Calex-18

```
       AIC      BIC    logLik
  338.9701 360.7892 - 161.4850

Random effects:

        (Intercept)  Residual
StdDev:   0.6078856 0.7251879

                      B             SE(B)   DF      t             p
(Intercept)           - 0.65        0.81    66      - 0.80        0.43
Sex                     0.27        0.16    66        1.73        0.09
Site quality          - 0.64        0.33    66      - 1.93        0.06
Hatch date              0.01        0.01    66        1.01        0.31
Length of BP care     - 0.01        0.01    66      - 0.72        0.48
Ho Calex-18           - 0.11        0.25    66      - 0.42        0.68

Number of Observations: 119
Number of Groups: 48
```

## Calex-19

```
       AIC      BIC    logLik
  338.9796 360.7987 - 161.4898

Random effects:

        (Intercept)  Residual
StdDev:   0.6065246 0.7264356

                      B             SE(B)   DF      t             p
(Intercept)           - 0.73        0.86    66      - 0.85        0.40
Sex                     0.27        0.16    66        1.70        0.09
Site quality          - 0.65        0.33    66      - 1.96        0.05
Hatch date              0.01        0.01    66        0.99        0.33
Length of BP care     - 0.01        0.01    66      - 0.72        0.47
Ho Calex-19             0.01        0.27    66        0.02        0.98

Number of Observations: 119
Number of Groups: 48
```

## Calex-22

```
      AIC      BIC    logLik
  339.463 361.2821 - 161.7315

Random effects:

        (Intercept) Residual
StdDev:   0.6030333 0.725418

                      B             SE(B)   DF      t             p
(Intercept)           - 0.62        0.80    66      - 0.77        0.44
Sex                     0.27        0.16    66        1.74        0.09
Site quality          - 0.61        0.33    66      - 1.85        0.07
Hatch date              0.01        0.01    66        0.95        0.35
Length of BP care     - 0.01        0.01    66      - 0.71        0.49
Ho Calex-22           - 0.12        0.16    66      - 0.74        0.46

Number of Observations: 119
Number of Groups: 48
```

## Calex-23

```
       AIC      BIC    logLik
  337.3307 359.1498 - 160.6653

Random effects:

        (Intercept)  Residual
StdDev:   0.6237038 0.7140377

                      B             SE(B)   DF      t             p
(Intercept)           - 0.35        0.86    66      - 0.40        0.69
Sex                     0.26        0.15    66        1.69        0.10
Site quality          - 0.61        0.33    66      - 1.84        0.07
Hatch date              0.01        0.01    66        0.94        0.35
Length of BP care     - 0.01        0.01    66      - 0.70        0.49
Ho Calex-23           - 0.37        0.31    66      - 1.21        0.23

Number of Observations: 119
Number of Groups: 48
```

## Uncorrected heterozygosity across all loci

```
       AIC      BIC    logLik
  336.7694 358.5885 - 160.3847

Random effects:

        (Intercept)  Residual
StdDev:   0.6060294 0.7252515

                      B             SE(B)   DF      t             p
(Intercept)           - 0.44        0.93    66      - 0.47        0.64
Sex                     0.28        0.16    66        1.76        0.08
Site quality          - 0.64        0.33    66      - 1.92        0.06
Hatch date              0.01        0.01    66        1.03        0.30
Length of BP care     - 0.01        0.01    66      - 0.70        0.48
Ho ALL                - 0.40        0.70    66      - 0.57        0.57

Number of Observations: 119
Number of Groups: 48
```

## 'Internal relatedness' across all loci

```
       AIC      BIC    logLik
  337.0125 358.8316 - 160.5063

Random effects:

        (Intercept)  Residual
StdDev:   0.6035803 0.7258433

                      B             SE(B)   DF      t             p
(Intercept)           - 0.77        0.79    66      - 0.97        0.34
Sex                     0.28        0.16    66        1.76        0.08
Site quality          - 0.64        0.33    66      - 1.94        0.06
Hatch date              0.01        0.01    66        1.04        0.30
Length of BP care     - 0.01        0.01    66      - 0.70        0.49
IR ALL                  0.38        0.60    66        0.64        0.53

Number of Observations: 119
Number of Groups: 48
```

## 'Homozygosity by loci' across all loci

```
       AIC      BIC    logLik
  336.7978 358.6169 - 160.3989

Random effects:

        (Intercept)  Residual
StdDev:   0.6062886 0.7254784

                      B             SE(B)   DF      t             p
(Intercept)           - 0.82        0.82    66      - 1.01        0.32
Sex                     0.28        0.16    66        1.75        0.08
Site quality          - 0.64        0.33    66      - 1.93        0.06
Hatch date              0.01        0.01    66        1.03        0.31
Length of BP care     - 0.01        0.01    66      - 0.71        0.48
HL ALL                  0.36        0.72    66        0.50        0.62

Number of Observations: 119
Number of Groups: 48
```

# Mean residuals of body mass growth – Random factor: Social family

## Environmental variables only

```
       AIC     BIC    logLik
  448.3176 467.471 - 217.1588

Random effects:

        (Intercept) Residual
StdDev:   0.9268386 1.219210
```

| | B | SE(B) | DF | t | p |
|---|---|---|---|---|---|
| (Intercept) | - 2.56 | 1.28 | 71 | - 2.00 | 0.05 |
| Sex | 0.65 | 0.25 | 71 | 2.56 | 0.01 |
| Site quality | - 1.70 | 0.62 | 43 | - 2.76 | 0.01 |
| Hatch date | 0.03 | 0.01 | 71 | 1.80 | 0.08 |
| Length of BP care | 0.01 | 0.02 | 43 | 0.38 | 0.71 |

```
Number of Observations: 119
Number of Groups: 46
```

## Calex-01

```
      AIC      BIC   logLik
  449.216 471.0351 - 216.608

Random effects:

        (Intercept) Residual
StdDev:   0.8906352 1.229916
```

| | B | SE(B) | DF | t | p |
|---|---|---|---|---|---|
| (Intercept) | - 2.25 | 1.28 | 70 | - 1.75 | 0.08 |
| Sex | 0.68 | 0.26 | 70 | 2.66 | 0.01 |
| Site quality | - 1.71 | 0.60 | 43 | - 2.85 | 0.01 |
| Hatch date | 0.03 | 0.01 | 70 | 1.86 | 0.07 |
| Length of BP care | 0.01 | 0.02 | 43 | 0.39 | 0.69 |
| Ho Calex-01 | - 0.42 | 0.37 | 70 | - 1.13 | 0.26 |

```
Number of Observations: 119
Number of Groups: 46
```

## Calex-02

```
       AIC      BIC    logLik
  450.5057 472.3248 - 217.2528

Random effects:

        (Intercept) Residual
StdDev:    0.921232 1.227706
```

| | B | SE(B) | DF | t | p |
|---|---|---|---|---|---|
| (Intercept) | - 2.56 | 1.28 | 70 | - 2.00 | 0.05 |
| Sex | 0.64 | 0.26 | 70 | 2.49 | 0.02 |
| Site quality | - 1.69 | 0.61 | 43 | - 2.75 | 0.01 |
| Hatch date | 0.02 | 0.02 | 70 | 1.73 | 0.09 |
| Length of BP care | 0.01 | 0.02 | 43 | 0.39 | 0.70 |
| Ho Calex-02 | 0.06 | 0.36 | 70 | 0.18 | 0.86 |

```
Number of Observations: 119
Number of Groups: 46
```

## Calex-04

```
       AIC      BIC    logLik
  450.3773 472.1964 - 217.1886

Random effects:

        (Intercept) Residual
StdDev:     0.91361 1.229200

                      B             SE(B)   DF      t             p
(Intercept)           - 2.63        1.28    70      - 2.05        0.04
Sex                     0.65        0.26    70        2.52        0.01
Site quality          - 1.69        0.61    43      - 2.76        0.01
Hatch date              0.03        0.01    70        1.76        0.08
Length of BP care       0.01        0.02    43        0.35        0.73
Ho Calex-04             0.16        0.35    70        0.45        0.66

Number of Observations: 119
Number of Groups: 46
```

## Calex-05

```
       AIC      BIC    logLik
  450.6963 472.5154 - 217.3481

Random effects:

        (Intercept) Residual
StdDev:   0.9298642 1.224636

                      B             SE(B)   DF      t             p
(Intercept)           - 2.54        1.29    70      - 1.98        0.05
Sex                     0.66        0.26    70        2.55        0.01
Site quality          - 1.70        0.61    43      - 2.75        0.01
Hatch date              0.03        0.02    70        1.80        0.08
Length of BP care       0.01        0.02    43        0.40        0.69
Ho Calex-05           - 0.07        0.32    70      - 0.22        0.83

Number of Observations: 119
Number of Groups: 46
```

## Calex-08

```
       AIC     BIC    logLik
  450.1729 471.992 - 217.0865

Random effects:

        (Intercept) Residual
StdDev:   0.9387046 1.217550

                      B             SE(B)   DF      t             p
(Intercept)           - 2.62        1.29    70      - 2.03        0.05
Sex                     0.64        0.26    70        2.50        0.01
Site quality          - 1.68        0.62    43      - 2.70        0.01
Hatch date              0.03        0.02    70        1.68        0.10
Length of BP care       0.01        0.02    43        0.44        0.66
Ho Calex-08             0.25        0.31    70        0.81        0.42

Number of Observations: 119
Number of Groups: 46
```

## Calex-12

```
       AIC      BIC    logLik
  446.2865 468.1056 - 215.1433

Random effects:

        (Intercept) Residual
StdDev:   0.9220685  1.19741

                      B             SE(B)   DF      t             p
(Intercept)           - 2.07        1.29    70      - 1.61        0.11
Sex                     0.62        0.25    70        2.48        0.02
Site quality          - 1.80        0.61    43      - 2.94        0.01
Hatch date              0.03        0.01    70        1.86        0.07
Length of BP care       0.01        0.02    43        0.45        0.65
Ho Calex-12           - 0.67        0.31    70      - 2.14        0.036

Number of Observations: 119
Number of Groups: 46
```

## Calex-14

```
       AIC      BIC    logLik
  450.2158 472.0349 - 217.1079

Random effects:

        (Intercept) Residual
StdDev:   0.9267798 1.226012

                      B             SE(B)   DF      t             p
(Intercept)           - 2.55        1.43    70      - 1.78        0.08
Sex                     0.65        0.26    70        2.50        0.01
Site quality          - 1.70        0.62    43      - 2.73        0.01
Hatch date              0.03        0.02    70        1.76        0.08
Length of BP care       0.01        0.02    43        0.38        0.71
Ho Calex-14           - 0.002       0.42    70      - 0.01        1

Number of Observations: 119
Number of Groups: 46
```

## Calex-18

```
       AIC      BIC    logLik
  450.2384 472.0575 - 217.1192

Random effects:

        (Intercept) Residual
StdDev:    0.933314 1.223751

                      B             SE(B)   DF      t             p
(Intercept)           - 2.57        1.31    70      - 1.97        0.05
Sex                     0.65        0.26    70        2.54        0.01
Site quality          - 1.70        0.62    43      - 2.74        0.01
Hatch date              0.03        0.02    70        1.77        0.08
Length of BP care       0.01        0.02    43        0.38        0.71
Ho Calex-18             0.03        0.42    70        0.07        0.95

Number of Observations: 119
Number of Groups: 46
```

## Calex-19

```
      AIC      BIC    logLik
  449.4988 471.3179 - 216.7494

Random effects:

        (Intercept) Residual
StdDev:   0.9277359 1.221545
```

| | B | SE(B) | DF | t | p |
|---|---|---|---|---|---|
| (Intercept) | - 2.11 | 1.40 | 70 | - 1.50 | 0.14 |
| Sex | 0.62 | 0.26 | 70 | 2.40 | 0.02 |
| Site quality | - 1.69 | 0.62 | 43 | - 2.73 | 0.01 |
| Hatch date | 0.03 | 0.02 | 70 | 1.68 | 0.10 |
| Length of BP care | 0.01 | 0.02 | 43 | 0.40 | 0.69 |
| Ho Calex-19 | - 0.35 | 0.44 | 70 | - 0.78 | 0.44 |

```
Number of Observations: 119
Number of Groups: 46
```

## Calex-22

```
      AIC      BIC    logLik
  450.6374 472.4565 - 217.3187

Random effects:

        (Intercept) Residual
StdDev:   0.9375095 1.219060
```

| | B | SE(B) | DF | t | p |
|---|---|---|---|---|---|
| (Intercept) | - 2.41 | 1.31 | 70 | - 1.84 | 0.07 |
| Sex | 0.65 | 0.26 | 70 | 2.54 | 0.01 |
| Site quality | - 1.66 | 0.62 | 43 | - 2.66 | 0.01 |
| Hatch date | 0.03 | 0.02 | 70 | 1.75 | 0.09 |
| Length of BP care | 0.01 | 0.02 | 43 | 0.41 | 0.68 |
| Ho Calex-22 | - 0.19 | 0.27 | 70 | - 0.70 | 0.49 |

```
Number of Observations: 119
Number of Groups: 46
```

## Calex-23

```
      AIC      BIC    logLik
  446.7882 468.6073 - 215.3941

Random effects:

        (Intercept) Residual
StdDev:   0.9680568 1.191355
```

| | B | SE(B) | DF | t | p |
|---|---|---|---|---|---|
| (Intercept) | - 1.75 | 1.38 | 70 | - 1.27 | 0.21 |
| Sex | 0.64 | 0.25 | 70 | 2.55 | 0.01 |
| Site quality | - 1.62 | 0.63 | 43 | - 2.56 | 0.01 |
| Hatch date | 0.03 | 0.02 | 70 | 1.78 | 0.08 |
| Length of BP care | 0.01 | 0.02 | 43 | 0.35 | 0.73 |
| Ho Calex-23 | - 0.90 | 0.51 | 70 | - 1.78 | 0.08 |

```
 Correlation:

Number of Observations: 119
Number of Groups: 46
```

## Uncorrected heterozygosity across all loci

```
       AIC      BIC    logLik
  446.5970 468.4162 - 215.2985

Random effects:

        (Intercept) Residual
StdDev:   0.9482073 1.206821

                      B             SE(B)   DF     t             p
(Intercept)           - 1.58        1.48    70     - 1.07        0.29
Sex                     0.66        0.25    70       2.62        0.01
Site quality          - 1.68        0.62    43     - 2.69        0.01
Hatch date              0.03        0.02    70       1.89        0.06
Length of BP care       0.01        0.02    43       0.42        0.68
Ho ALL                - 1.42        1.06    70     - 1.33        0.19

Number of Observations: 119
Number of Groups: 46
```

## 'Internal relatedness' across all loci

```
       AIC      BIC    logLik
  446.5697 468.3888 - 215.2848

Random effects:

        (Intercept) Residual
StdDev:   0.9505437 1.203741

                      B             SE(B)   DF     t             p
(Intercept)           - 2.75        1.30    70     - 2.11        0.04
Sex                     0.66        0.25    70       2.60        0.01
Site quality          - 1.69        0.62    43     - 2.72        0.01
Hatch date              0.03        0.02    70       1.90        0.06
Length of BP care       0.01        0.02    43       0.43        0.67
IR ALL                  1.33        0.91    70       1.46        0.15

Number of Observations: 119
Number of Groups: 46
```

## 'Homozygosity by loci' across all loci

```
      AIC      BIC    logLik
  446.483 468.3021 - 215.2415

Random effects:

        (Intercept) Residual
StdDev:   0.9504506 1.205548

                      B             SE(B)   DF     t             p
(Intercept)           - 3.00        1.33    70     - 2.25        0.03
Sex                     0.66        0.25    70       2.61        0.01
Site quality          - 1.68        0.62    43     - 2.69        0.01
Hatch date              0.03        0.02    70       1.89        0.06
Length of BP care       0.01        0.02    43       0.41        0.68
HL ALL                  1.48        1.09    70       1.36        0.18

Number of Observations: 119
Number of Groups: 46
```

# Mean residuals of body mass growth – Random factor: Biological family

## Environmental variables only

```
      AIC      BIC   logLik
  454.536 473.6894 - 220.268

Random effects:

        (Intercept) Residual
StdDev:   0.8125074 1.290034

                      B             SE(B)   DF      t             p
(Intercept)           - 2.34        1.21    67      - 1.93        0.06
Sex                     0.58        0.27    67        2.17        0.03
Site quality          - 1.48        0.51    67      - 2.87        0.01
Hatch date              0.03        0.01    67        1.84        0.07
Length of BP care     - 0.01        0.02    67      - 0.19        0.85

Number of Observations: 119
Number of Groups: 48
```

## Calex-01

```
       AIC      BIC    logLik
  454.6786 476.4977 - 219.3393

Random effects:

        (Intercept) Residual
StdDev:    0.778657 1.296068

                      B             SE(B)   DF      t             p
(Intercept)           - 1.98        1.22    66      - 1.63        0.11
Sex                     0.63        0.27    66        2.36        0.02
Site quality          - 1.51        0.51    66      - 2.97        <0.01
Hatch date              0.03        0.01    66        1.91        0.06
Length of BP care       0           0.02    66      - 0.08        0.93
Ho Calex-01           - 0.54        0.39    66      - 1.38        0.17

Number of Observations: 119
Number of Groups: 48
```

## Calex-02

```
       AIC      BIC    logLik
  456.5645 478.3836 - 220.2823

Random effects:

        (Intercept) Residual
StdDev:   0.8056234 1.299071

                      B             SE(B)   DF      t             p
(Intercept)           - 2.35        1.21    66      - 1.94        0.06
Sex                     0.57        0.27    66        2.09        0.04
Site quality          - 1.47        0.51    66      - 2.86        0.01
Hatch date              0.02        0.01    66        1.72        0.09
Length of BP care       0           0.02    66      - 0.14        0.89
Ho Calex-02             0.10        0.38    66        0.26        0.80

Number of Observations: 119
Number of Groups: 48
```

## Calex-04

```
       AIC      BIC    logLik
  456.3627 478.1818 - 220.1814

Random effects:

        (Intercept) Residual
StdDev:   0.7914844 1.302644
```

| | B | SE(B) | DF | t | p |
|---|---|---|---|---|---|
| (Intercept) | - 2.45 | 1.22 | 66 | - 2.01 | 0.05 |
| Sex | 0.58 | 0.27 | 66 | 2.15 | 0.03 |
| Site quality | - 1.47 | 0.51 | 66 | - 2.87 | 0.01 |
| Hatch date | 0.03 | 0.01 | 66 | 1.80 | 0.08 |
| Length of BP care | 0 | 0.02 | 66 | - 0.19 | 0.85 |
| Ho Calex-04 | 0.21 | 0.38 | 66 | 0.54 | 0.59 |

```
Number of Observations: 119
Number of Groups: 48
```

## Calex-05

```
       AIC      BIC    logLik
  456.7722 478.5913 - 220.3861

Random effects:

        (Intercept) Residual
StdDev:   0.8165803 1.294714
```

| | B | SE(B) | DF | t | p |
|---|---|---|---|---|---|
| (Intercept) | - 2.33 | 1.22 | 66 | - 1.92 | 0.06 |
| Sex | 0.59 | 0.27 | 66 | 2.19 | 0.03 |
| Site quality | - 1.48 | 0.52 | 66 | - 2.87 | 0.01 |
| Hatch date | 0.03 | 0.01 | 66 | 1.86 | 0.07 |
| Length of BP care | 0 | 0.02 | 66 | - 0.14 | 0.89 |
| Ho Calex-05 | - 0.12 | 0.33 | 66 | - 0.35 | 0.73 |

```
Number of Observations: 119
Number of Groups: 48
```

## Calex-08

```
      AIC      BIC    logLik
  456.877 478.6961 - 220.4385

Random effects:

        (Intercept) Residual
StdDev:   0.8130036 1.296200
```

| | B | SE(B) | DF | t | p |
|---|---|---|---|---|---|
| (Intercept) | - 2.37 | 1.22 | 66 | - 1.95 | 0.06 |
| Sex | 0.58 | 0.27 | 66 | 2.13 | 0.04 |
| Site quality | - 1.48 | 0.52 | 66 | - 2.86 | 0.01 |
| Hatch date | 0.03 | 0.01 | 66 | 1.79 | 0.08 |
| Length of BP care | 0 | 0.02 | 66 | - 0.17 | 0.86 |
| Ho Calex-08 | 0.10 | 0.32 | 66 | 0.30 | 0.76 |

```
Number of Observations: 119
Number of Groups: 48
```

## Calex-12

```
       AIC      BIC    logLik
  454.3582 476.1773 - 219.1791

Random effects:

        (Intercept) Residual
StdDev:      0.7412 1.304761

                      B             SE(B)   DF     t             p
(Intercept)           - 1.96        1.19    66     - 1.65        0.10
Sex                     0.57        0.26    66       2.14        0.04
Site quality          - 1.58        0.50    66     - 3.14        <0.01
Hatch date              0.03        0.01    66       1.96        0.05
Length of BP care       0           0.02    66     - 0.18        0.85
Ho Calex-12           - 0.54        0.33    66     - 1.64        0.11

Number of Observations: 119
Number of Groups: 48
```

## Calex-14

```
       AIC     BIC    logLik
  455.6619 477.481 - 219.8310

Random effects:

        (Intercept) Residual
StdDev:   0.8281707 1.288030

                      B             SE(B)   DF     t             p
(Intercept)           - 2.85        1.43    66     - 1.99        0.05
Sex                     0.62        0.27    66       2.25        0.03
Site quality          - 1.53        0.52    66     - 2.92        <0.01
Hatch date              0.03        0.01    66       1.93        0.06
Length of BP care       0           0.02    66     - 0.21        0.84
Ho Calex-14             0.34        0.49    66       0.69        0.49

Number of Observations: 119
Number of Groups: 48
```

## Calex-18

```
       AIC      BIC    logLik
  456.4093 478.2284 - 220.2047

Random effects:

        (Intercept) Residual
StdDev:   0.8208767 1.293676

Fixed effects: W.rsum ~ C.sex + Q.site + Hatch date + Length of BP care + Ho Calex-18
                      B             SE(B)   DF     t             p
(Intercept)           - 2.40        1.25    66     - 1.92        0.06
Sex                     0.58        0.27    66       2.15        0.04
Site quality          - 1.48        0.52    66     - 2.86        0.01
Hatch date              0.03        0.01    66       1.81        0.07
Length of BP care       0           0.02    66     - 0.19        0.85
Ho Calex-18             0.09        0.42    66       0.22        0.83

Number of Observations: 119
Number of Groups: 48
```

## Calex-19

```
       AIC     BIC    logLik
  455.6799 477.499 - 219.8399

Random effects:

        (Intercept) Residual
StdDev:    0.803257 1.296190

                      B             SE(B)   DF      t             p
(Intercept)           - 1.91        1.33    66      - 1.44        0.15
Sex                     0.56        0.27    66        2.08        0.04
Site quality          - 1.47        0.51    66      - 2.87        0.01
Hatch date              0.02        0.01    66        1.74        0.09
Length of BP care       0           0.02    66      - 0.15        0.88
Ho Calex-19           - 0.35        0.45    66      - 0.77        0.44

Number of Observations: 119
Number of Groups: 48
```

## Calex-22

```
       AIC      BIC    logLik
  456.6955 478.5146 - 220.3478

Random effects:

        (Intercept) Residual
StdDev:   0.8279487 1.287510

                      B             SE(B)   DF      t             p
(Intercept)           - 2.16        1.24    66      - 1.74        0.09
Sex                     0.58        0.27    66        2.18        0.03
Site quality          - 1.42        0.52    66      - 2.72        0.01
Hatch date              0.02        0.01    66        1.77        0.08
Length of BP care       0           0.02    66      - 0.16        0.87
Ho Calex-22           - 0.21        0.28    66      - 0.75        0.46


Number of Observations: 119
Number of Groups: 48
```

## Calex-23

```
       AIC      BIC    logLik
  452.3662 474.1853 - 218.1831

Random effects:

        (Intercept) Residual
StdDev:   0.8755868 1.248410

                      B             SE(B)   DF      t             p
(Intercept)           - 1.33        1.34    66      - 0.99        0.32
Sex                     0.55        0.26    66        2.10        0.04
Site quality          - 1.38        0.52    66      - 2.63        0.01
Hatch date              0.02        0.01    66        1.73        0.09
Length of BP care       0           0.02    66      - 0.17        0.86
Ho Calex-23           - 1.00        0.51    66      - 1.97        0.053

Number of Observations: 119
Number of Groups: 48
```

## Uncorrected heterozygosity across all loci

```
       AIC      BIC    logLik
  452.6686 474.4877 - 218.3343

Random effects:

        (Intercept) Residual
StdDev:   0.8284884 1.279257

                      B             SE(B)   DF      t             p
(Intercept)           - 1.26        1.46    66      - 0.86        0.39
Sex                     0.61        0.27    66        2.28        0.03
Site quality          - 1.44        0.52    66      - 2.78        0.01
Hatch date              0.03        0.01    66        1.93        0.06
Length of BP care       0           0.02    66      - 0.14        0.89
Ho ALL                - 1.54        1.17    66      - 1.32        0.19

Number of Observations: 119
Number of Groups: 48
```

## 'Internal relatedness' across all loci

```
       AIC      BIC    logLik
  452.9306 474.7497 - 218.4653

Random effects:

        (Intercept) Residual
StdDev:    0.825181 1.280111

                      B             SE(B)   DF      t             p
(Intercept)           - 2.50        1.22    66      - 2.05        0.05
Sex                     0.60        0.27    66        2.25        0.03
Site quality          - 1.46        0.52    66      - 2.83        0.01
Hatch date              0.03        0.01    66        1.93        0.06
Length of BP care       0           0.02    66      - 0.13        0.89
IR ALL                  1.33        1.00    66        1.33        0.19

Number of Observations: 119
Number of Groups: 48
```

## 'Homozygosity by loci' across all loci

```
       AIC      BIC    logLik
  452.6633 474.4824 - 218.3317

Random effects:

        (Intercept) Residual
StdDev:    0.827728 1.279827

                      B             SE(B)   DF      t             p
(Intercept)           - 2.75        1.26    66      - 2.20        0.03
Sex                     0.61        0.27    66        2.27        0.03
Site quality          - 1.44        0.51    66      - 2.79        0.01
Hatch date              0.03        0.01    66        1.93        0.06
Length of BP care       0           0.02    66      - 0.15        0.88
HL ALL                  1.55        1.20    66        1.30        0.20

Number of Observations: 119
Number of Groups: 48
```

Chapter VI

# Conclusions

My PhD results have important implications for understanding evolutionary biology, systematics, conservation biology and population genetics of shorebirds. Specifically,

- I provided powerful sets of microsatellite markers for several shorebird species (Chapters II and III). I showed that genomic resources of model organisms can help to improve molecular markers and make them applicable to a large range of non-model organisms (Chapter III).
- I demonstrated that the degree of conservation of genomic regions in which markers are located is important for marker utility. The results of marker development support the proposition that the utility of molecular makers depends on the genetic diversity of populations. In Eurasian Kentish plovers most markers were polymorphic, and the large number of alleles and high levels of heterozygosity at most loci suggest that populations are unusually genetically diverse (Chapters II-V). However, I also showed that different plover populations vary in their genetic diversity and high/low variability at nuclear markers is mirrored by high/low variability at mitochondrial markers (Chapter IV). Therefore, low genetic diversity should be seen as feature of the recent phylogenetic history of populations, but not as a general feature of shorebirds.
- Using mitochondrial and microsatellite markers I investigated the phylogenetic relationships between populations of Kentish plovers, snowy plovers and white-fronted plovers. My major conclusion is that the present shorebird phylogeny, which is largely based on morphological characteristics, has serious shortcomings (Chapter IV). Some species are not monophyletic and careful phylogeographic analyses need to be conducted to resolve the interpopulation relationships. The current taxonomic status of the Kentish plover needs to be revised, and the American populations ('snowy plovers') should be recognised as a separate species.
- Finally, I showed that offspring survival of Kentish plovers is influenced by their genetics since there are beneficial and detrimental allele combinations (Chapter V). Heterozygosity-fitness correlations between microsatellite markers and chick survival suggest that there is no genomewide beneficial effect of heterozygosity on fitness. Survival patterns associated with different loci show that both overdominance (heterozygosity is beneficial) and underdominance (heterozygosity is harmful) may strongly influence survival during juvenile development.

The phylogeography of all three North American snowy plover subspecies has been recently investigated. Using a subset of the microsatellite markers described in Chapter II, Funk *et al.* (2007) found evidence for genetic differentiation between the subspecies. However, there was a striking lack of population differentiation between isolated population fragments within each subspecies similar to my findings for populations within the *C. a. alexandrinus* subspecies (Chapter IV). The observed genetic diversity in all three American subspecies was low in comparison to the diversity harboured in nominate subspecies *alexandrinus*. This suggests that different conservation measures need to be considered for the conservation management of American and Eurasian plover populations.

Recent molecular ecological studies of two other shorebird species, dunlin *Calidris alpina* and great snipe *Gallinago media* revealed further interesting messages for conservation. First, in dunlins three of 11 named subspecies have been defined according to differences in morphological and behavioural traits such as bill length, wing length, plumage, time of breeding and migration direction (*C. a. alpina, C. a. arctica, C. a. schinzii,* Soikkeli 1966, Cramp & Simmons 1983, del Hoyo *et al.* 1996). However, the three subspecies share mitochondrial haplotypes and microsatellite alleles and their genetics reveal a clinal differentiation with the frequencies of mitochondrial haplotypes and microsatellites alleles changing gradually over large geographic distances (Marthinsen *et al.* 2007). This provides new challenges in defining conservation management units in dunlins since the Baltic Sea populations (part of the subspecies *schinzii*) are severely declining, but *schinzii* dunlins are genetically very similar to *alpina* dunlins whose populations are less threatened. Second, in the great snipe that breeds in Northern Europe no population differentiation between Eastern and Western demes was found using putatively neutral markers such as microsatellites. However, markers derived from major histocompatibility complex genes that are likely to be under selection revealed differentiation between eastern and western populations. These patterns could not be explained by isolation by distance alone, but suggest local adaptations to different parasite challenges (Ekblom *et al.* 2007). The case of the great snipe highlights a problem for conservation genetics; appropriate management practice using conservation genetics must be based on a variety of different genetic markers, however, funding (and appropriate genetic markers) are often severly limited.

The results of my analysis on marker based heterozygosity and fitness are in the line with recent studies that suggest that heterozygosity-fitness correlations may reflect a

role of certain genomic regions with which the marker is linked (local effect) rather than an effect of the microsatellite itself (direct effect). However, functional studies are needed to discriminate between these two hypotheses. The observed antagonistic effects of underdominance and overdominance at different microsatellite loci provide one reason why positive genomewide heterozygosity-fitness correlations are absent or weak and why marker based estimates of inbreeding coefficients often fail to explain inbreeding depression (Slate *et al.* 2004, Markert *et al.* 2004, Pemberton *et al.* 2004). It also shows that offspring survival does not depend on environmental conditions and parental care alone, but intrinsic factors of the precocial chicks may also influence survival until fledging.

## Future directions

The PhD projects have generated a number of interesting questions that should be addressed by future research. Three research lines appear to be the most promising.

**1. Phylogeography of a cosmopolitan shorebird – the colonisation of the Americas**

Kentish/snowy plovers are unusual among shorebirds in that they have a wide geographic distribution in Europe, North Africa, Central and East Asia, and the Americas. Snowy plovers are not only genetically different, but they also exhibit much lower genetic variation than the Eurasian nominal subspecies *C. alexandrinus alexandrinus* (Funk *et al.* 2007, Chapter IV). There may be two reasons for the low genetic variation in snowy plovers. First, snowy plovers went through a bottleneck during recent glaciations when the population was forced into small refugia (Wenink *et al.* 1994, Baker 2006). Second, a small number of Kentish plovers colonized the American continent from Europe, North Africa or Asia, and descendants of these founders spread across North, Central and South America. These hypotheses can be tested by sampling Pacific and Atlantic populations from Eurasia and Africa and retracting possible colonisation routes by comparing allele frequencies of potential source populations with the frequencies present in American populations.

**2. Phylogeography of Kentish plover superspecies**

The phylogeographic approach can be extended to additional plover populations using the same molecular markers. Kentish plover, snowy plover, white-fronted plover *C. marginatus*, red-capped plover *C. ruficapillus* and Javan plover *C. javanicus* form an allopatric superspecies complex. The populations of the current species *C. alexandrinus*

differ strikingly in their genetic variation (Chapter IV, Funk *et al.* 2007). Coalescent analyses will determine the origin of the superspecies (Rosenberg & Nordborg 2002, Beaumont 2004). An appropriate phylogeographic analysis will not only clarify the recent contentious phylogenetic relationships between Kentish plover subspecies, but may reveal further cryptic species and therefore identify better conservation management units (Kennerly *et al.* 2008, del Hoyo *et al.* 1996). Interestingly, although birds have always been the model for speciation processes for decades (Price 2007), the systematics of many avian groups are still unsatisfactorily resolved (Hackett *et al.* 2008). For example, more than 90 cryptic species have been discovered over the last 30 years (Pfenniger & Schwenk 2007). This may have implications for conservation: two closely related species which are by some authors included into the superspecies (see del Hoyo *et al.* 1996), Malaysian *C. peronii* and Java plover *C. javanicus* are classified as 'Near Threatened' by IUCN.

**3. Candidate genes for breeding system evolution**

Plover populations differ greatly in their breeding systems (Warriner *et al.* 1986, Lessells 1984, Székely & Williams 1995, Lloyd 2007). Coupled with behavioural studies of the breeding systems, a thorough phylogeographic analysis may help to improve our understanding of the evolution of breeding systems. Comparative research on breeding system evolution has revealed striking phylogenetic and ecological patterns in shorebirds (Székely *et al.* 2004, Thomas *et al.* 2006). However, little is known about genetic bases of breeding system evolution. Interestingly, microsatellite alleles are correlated with different breeding systems in voles. A single microsatellite located in the *cis* regulatory region of the vasopressin *V1a* receptor plays a significant role in determining social phenotypes (Hammock & Young 2004). Different alleles predict different distribution patterns of *V1a* receptors in vole brains and also different individual male social behaviour. Males with longer microsatellite alleles showed more behaviours associated with polygyny than males with shorter alleles, who showed behaviours associated with monogamy (Hammock & Young 2005). However, a phylogenetic comparative study using 25 rodent species did not find evidence for an exclusive association of short microsatellite alleles with social monogamy, conversely males of two species that are largely monogamous showed genotypes with long microsatellite alleles in the regulatory region of the vasopressin receptor (Fink *et al.* 2007). One hypothesis that remains to be tested is whether genetic diversity is important for mating behaviour (Young & Hammock 2007) and shorebirds provide an excellent

system to carry out such a test. The diversity of shorebird breeding systems exceeds the variation of breeding system among voles and most other mammals. Markers for conserved microsatellites located in avian genes can be developed using the available chicken *Gallus gallus* and zebra finch *Taeniopygia guttata* genome databases. Conserved microsatellite markers provide the first candidate loci to examine correlations between alleles and behaviour, whilst non-conserved markers can be used to estimate genetic diversity. Transitions between breeding systems have occurred several times in the phylogenetic history of shorebirds (Székely & Reynolds 1995). A phylogeographic analysis in the superspecies Kentish plover, a species with exceptional variability of breeding behaviours across populations, could show whether breeding system changes are reflected in changes of genetic diversity and/or allele frequencies between populations.

The diversity of Kentish plover reproductive behaviour provides many clear examples to understand social evolution and sexual selection (Székely *et al.* 2006). Recent progress in the development of new molecular and bioinformatic techniques has made it feasible to obtain gene expression patterns in non-model organisms (Blow 2007, Ellegren 2008). This provides new opportunities to determine the genetic bases of social behaviour. However, genomic studies alone and snapshots on gene-expression are not enough to understand selection pressures acting on different behaviour, they need to be combined with observational and experimental long term studies of natural populations.

Two important measures that can be addressed by such ecological studies are repeatability and heritability. Repeated behavioural sampling from the same individual can provide a measure for the consistency of behaviour. Repeatability can be used as surrogate to estimate the plasticity of behaviour (Appendix II) and may also provide the upper limit for heritability of behaviour (Falconer & MacKay 1996). Heritability of many traits can be determined by sampling behaviour in relatives and exploring the changes of behaviour along pedigrees by mixed models with restricted maximum likelihood methods (Kruuk 2004, Postma & Charmantier 2007). Heritabilities and the extent of genetic variation held in populations are not only important for determining the genetic base of behaviour, but also to understand the ability of populations to adapt to changes in the environment. Changes in today's world are often caused or magnified by human activities.

### 4. Candidate genes for fitness

The results from the microsatellite analyses show that different genotypes are associated with different aspects of fitness (Chapter V). The genomic locations of the conserved markers provide a starting point for an investigation into the genes associated with the observed fitness effects. Genomic databases, such as chicken or zebra finch genome database can be used to identify candidate loci in the vicinity of microsatellite markers which then can be examined for the degree of linkage with the markers and their role in fitness. Heterozygosity-fitness correlations need also to be explored in adults to see whether reproductive success or longevity is associated with any of these loci. Examinations may also include stratification tests to identify beneficial and detrimental alleles and allele combinations.

This PhD has provided the basic tools to investigate the molecular ecology of Kentish plovers and other shorebirds. Molecular ecology in shorebirds is still in its infancy, but as this PhD and recent similar studies have shown, there are exciting perspectives.

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Appendix I

# Breeding ecology of Kentish Plover *Charadrius alexandrinus* in an extremely hot environment

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## Abstract

**Capsule.** Here we report the breeding behaviour of Kentish Plovers from the Arabian Peninsula, one of the hottest locations in their breeding distribution.

**Aims.** To investigate breeding ecology and parental behaviour of Kentish Plovers in an extremely hot environment. The Kentish Plover has an unusually diverse breeding system in which the frequencies of biparental care, female-only care and male-only care vary between populations. A common, but rarely tested, explanation for such a variation is local adaptation: birds exhibit social traits that are adaptive to their breeding environment.

**Methods.** A breeding population of approximately 200 pairs was investigated in 2005 and 2006 at Al Wathba Wetland which is located near Abu Dhabi in the United Arab Emirates.

**Results.** We found high nest site fidelity and low mate fidelity, and more biparental care in Al Wathba than in most temperate zone populations of Kentish Plover.

**Conclusions.** Our results are consistent with the argument that harsh environment can select for biparental care. However, further studies are warranted to distinguish between alternative hypotheses for the different distribution of social behaviours of breeding populations.

## Introduction

Waders (or shorebirds, approximately 350 species, Monroe & Sibley 1993) have a global distribution: they breed on all continents including Antarctica (del Hoyo et al. 1996, Kam et al. 2004). Successful breeding in vastly different environments such as Arctic tundra, temperate wetlands, deserts and tropical marshes requires special adaptations. These adaptations may influence fundamental aspects of birds' life including physiology, life histories and resilience to perturbations caused by humans.

There are two fundamental reasons for collecting data on behaviour and ecology of waders in different environments. First, waders exhibit an unusual diversity in their behaviour, ecology and life histories, with complex phylogenetic relationships between these traits (Bókony et al. 2003, Székely et al. 2004, Thomas & Székely 2005, Thomas et al. 2007). Studies have shown that the environment often shapes these traits, although a common limitation of such comparative studies is that life-history and ecological data are scanty from tropical and subtropical regions, where many waders breed. Second, to assess the status and trends of populations, we need baseline data on breeding ecology and the long-term sustainability of populations. Whilst waders feature prominently in studies of evolutionary ecology including migration, foraging ecology and breeding systems (see reviews by del Hoyo et al. 1996, Kam et al. 2004, Thomas et al. 2007), collecting data on their demography is difficult, due to their low breeding densities in transient habitats, the nomadic nature of breeding adults, and the high mobility of their young (but see McCulloch 1992, Sandercock 2003, Watson et al. 2006, Lloyd 2008, Zefania et al. 2008).

A powerful approach to investigate the influence of environment on breeding strategies is to focus on a single species that breeds under different environmental regimes, and look to see whether these different ecological conditions may evoke different social traits, for instance mating system and parental care. Here we focus on a widely distributed wader, the Kentish Plover *Charadrius alexandrinus*. This species has a vast geographic distribution in Eurasia from Cape Verde Islands in the west to Taiwan and Japan in the east, and they breed in open habitats on the north hemisphere between the 55° (N Germany) and 10° (Sri Lanka). Kentish Plovers are polygamous (either by the male or the female, Lessells 1984, Warriner et al. 1986, Blomqvist et al. 2002), and the chicks may be raised by a single parent or both parents. Kentish Plover is one of the few bird species in which several factors have been identified that may influence whether

one or both parents care for the young (Amat et al. 1999, Székely & Cuthill 1999, Székely et al. 1999, Kosztolányi et al. 2006). Extreme ambient environment may select for biparental care, which may induce a suit of changes in life history traits including persistence in breeding at the same site with the same mate (Lloyd 2008, Al-Rashidi et al. submitted).

We have three objectives in this study. First, to investigate breeding strategies of Kentish Plovers in a harsh desert environment where ground surface temperature often rises over 50 ºC, and occasionally reach 65 ºC (A. Kosztolányi pers. obs.). Given such demand on the parents to cool the eggs, we expected many nests to fail. Parental care, sexual selection and their relationship with the environment are controversial topics in evolutionary biology (Kokko & Jennions 2008), and empirical studies, like the one we present here, are needed to underpin (or disprove) theoretical arguments. Second, to protect chicks from scorching heat, both parents must cooperate to rear the young. Finally, if the breeding environment exhibits low variation across the year, one may expect pairs staying together between years and, due to a non-migratory life-style, they return to their former home range. Previous studies have investigated the breeding ecology of Kentish Plovers in Europe (Germany, Rittinghaus 1961; Hungary, Székely 1991; France, Lessells 1984; Spain, Fraga & Amat 1996; Turkey, Kosztolányi et al. 2006), although here we investigate Kentish Plovers in the southernmost population to date, the Arabian Peninsula. Previous account of Kentish Plovers in Abu Dhabi reported sightings, and the occurrences of chicks and nests (P. Hellyer, S. Aspinall pers. comm.), although no detailed study is available from any Eurasian population that breeds further south than Turkey.

## Methods

We investigated the Kentish Plovers at Al Wathba Wetland Reserve, about 40 km south-east of Abu Dhabi, United Arab Emirates (24° 15.5’ N, 54° 36.2’ E) in two years (2005: 23 March – 23 July; 2006: 26 April – 12 July). The Reserve is approximately 3.7 km × 1.9 km with total area about 450 ha; it is composed of water bodies surrounded by sand dunes. The Reserve is managed by Environment Agency – Abu Dhabi (EAD), and the area is surrounded by a fence that excludes large terrestrial predators (see detailed description by Khan & Javed 2004).

We used standard methodology to estimate survival of nests and broods, and chick growth rates (Kosztolányi et al. 2006, Székely et al. 2008). In short, we searched for nests by observing plovers from a hide or car, or by walking through the area. For each nest standard measurements were taken (Székely et al. 2008), and the fate of the clutch was assigned to one of the following categories: 'Hatched' – at least one egg hatched, 'Predated' – the eggs were depredated, 'Failed' – no eggs hatched (because the eggs were infertile or the embryo died in the egg and/or the parents deserted the nest), 'Unknown' – the fate of the nest was not followed or the eggs disappeared, but neither predation nor hatching was confirmed.

We set up nest camera systems to record the behaviour of parents at the nest. The system consisted of a small camera (Outdoorcam, Swann Communications Pty. Ltd.) positioned about 1 m from the nest, and a digital video recorder (MemoCam, Video Domain Technologies Ltd.) that recorded images on a memory card every 20 s. The camera had infrared lights for night images.

Adults were identified in the field by a unique combination of one metal and three colour rings. Downy chicks were ringed by two rings, one metal ring and a colour ring (the same colour within a family).

In 2005, broods were visited regularly after hatching, and at each visit the location of the family, the sex and number of attending parents and the number of chicks were recorded. If a parent was not present at the brood at two consecutive visits, we considered the parent as having deserted. Broods were followed until the chicks were 25 days old. For broods which hatched from unknown nest, the chicks were ringed at the first encounter, and we used their tarsus length to estimate hatching date (see Székely & Cuthill 1999). Chicks were recaptured to measure their left and right tarsus length (nearest 0.1mm). Growth rate of chicks was estimated by fitting a least-squares regression for each brood, and using the slope of this regression ($mm \times day^{-1}$). Tarsus growth is approximately linear in the Kentish Plover between hatching and age 25 days (Székely & Cuthill 1999).

During fieldwork whenever we encountered a colour-ringed plover we recorded its location, and noted its behaviour. We defined the home range of an individual as the convex hull of the points where the plover was observed in a given year. In home range

size calculations only those birds were included that were observed at least three different locations in a year. The distance that birds moved between years was calculated as the distance between the centroids of the two home range polygons. For plovers with several nests in a year we calculated the between-year nest distance using their first known nest in each year.

Statistical analyses were carried out using R 2.7.1 (R Development Core Team 2008). For non-parametric data we provide the median (M), the lower quartiles (LQ) and the upper quartiles (UQ).

## Results

Egg laying dates and incubation period

We found 317 nests in total in the two years. Out of 260 completed nests 163 had three eggs, 82 had two eggs and 15 had only one egg. Eggs were laid between 13 March and 24 June (2005), and 13 April and 20 June (2006). Most eggs were laid in April and May (Fig. 1). Note, however, when fieldwork started in 2005 we had already observed chicks. Based on the estimated ages of these chicks, egg laying may start as early as February, and it may last until late November, since S. Aspinall (pers. comm.) reported downy chicks from Al Wathba in December 2005.

23.4% and 19.4% of nests hatched in 2005 and 2006, respectively (Table 1). The fate of nests did not differ between years ($\chi^2$ test of homogeneity for hatched, predated and failed categories, $\chi^2 = 0.592$, df = 2, $P = 0.744$). Nest cameras identified Grey Monitors *Varanus griseus*, Red Foxes *Vulpes vulpes* and a Red-wattled Lapwing *Vanellus indicus* taking eggs (Fig. 2), and we found footprints of Grey Monitors around several predated nests. In many cases, however, the predator(s) did not leave footprints so that we could not identify them. Nests that hatched tended to be laid earlier (64.5 (50.5–81.0) days after 1 March, M (LQ–UQ), $n = 68$ nests) than unhatched (predated or failed) nests (71 (53–86) days after 1 March, $n = 130$ nests, Mann-Whitney test, $W = 3764.5$, $P = 0.087$).

We recorded the behaviour of parents for 1-3 days using camera systems ($n = 28$ nests). At each nest both parents incubated the eggs. The length of the incubation period was 25 (24–25) days, at $n = 7$ hatched nests we found before clutch completion).

Brood-rearing period

In 2005, 30 broods (15 hatched from known nest and 15 hatched from unknown nest) were checked regularly until the age of 25 days or until all chicks perished. 25 of these families fledged at least one chick successfully, and the probability of fledging decreased during the breeding season (Fig. 3, Generalized Linear Model with binomial error, $B = -0.20 \pm 0.09$, $P = 0.034$).

Tarsus growth of chicks was $0.42 \pm 0.03$ mm $\times$ day$^{-1}$ (mean $\pm$ se, $n = 30$ broods), and chicks hatched later in the season grew faster than chicks hatched earlier (least-squares regression, $B = 3.50 \times 10^{-3} \pm 1.18 \times 10^{-3}$ mm $\times$ day$^{-2}$, $P = 0.006$).

Biparental brood care, male-only brood care and female-only brood care were all observed in Al Wathba, and the distribution of care types tended to change with both hatching date and brood age (Fig. 4). We observed 15 female and 5 male desertions, whereas in 10 families both parents stayed with the chicks until the age of 25 days. Male desertion occurred 5.5 (3.5–5.5) days after hatching, whereas female desertion occurred 12.5 (4.3–17.5) days after hatching (Mann-Whitney test, $W = 52$, $P = 0.221$). Consistently with the changes in the distribution of care types, male desertion was more likely to occur in early broods (Fig. 5, Kruskal-Wallis test, $\chi^2 = 8.850$, df = 2, $P = 0.012$).

We observed five cases of sequential polygamy out of 7 males and 10 females that bred several times with known mates in a breeding season: one male in 2005 and four females (two in 2005 and two in 2006) renested with a different mate within the same breeding season. The polygynous male was tending alone two 22-day old chicks from his first nest on 29 April and his first mate was seen with an unringed male on 28 April. The male renested with an unringed female on 7 June. The first mate of this polygynous male renested with an unringed male on 21 May. The other polyandrous female in 2005 deserted her first brood on 24 April when the chicks were 14 days old, and laid a new clutch with an unringed male around 20 May. One polyandrous female in 2006 remated with a ringed male (her previous mate in 2005) after her first clutch was predated, however, her first mate was still alive and present in Al Wathba. The other polyandrous female in 2006 deserted her first brood on 27 May when the chicks were 14 days old, and then laid a new clutch with an unringed male on 5 June.

Mate fidelity and site fidelity

Both parents were ringed in 99 pairs in 2005. Out of these, the mate of 16 males and 12 females was identified in 2006. Six males (37.5%) out of the 16, and six females (50.0%) out of the 12 remated with the same bird in the next year (two out of these six females, however, had two mates in one of the years).

147 colour ringed adults were observed at the study site in 2006 out of 223 adults that were marked in 2005. The returning rate of males (70.0%, 77 out 110) did not differ from that of females (61.9%, 70 out 113, $\chi^2$ test of homogeneity, $\chi^2 = 1.271$, df = 1, $P = 0.260$).

Females tended to move larger distances between years than males, although the home ranges of sexes were not different (Table 2). Plovers tended to have smaller home ranges in 2005 (1.50 (0.19–4.75) ha than in 2006 (2.30 (0.49–8.11) ha; Wilcoxon matched-pairs test, $V = 305$, $n = 42$, $P = 0.068$).

We identified the nests of 38 adults in 2006 that were colour ringed in 2005. The locations of nests in 2005 were known for 34 out of 38 plovers, whilst the remaining four plovers were caught when they were tending the chicks, and thus their nest location was unknown (Fig. 6). The distance between nests in the two years was 92 (30–263) m. The between-year nest distances of females was not different (139 (70–688) m, $n = 13$ females) from that of males (70 (28–216) m, $n = 21$ males, Mann-Whitney test, $W = 175$, $P = 0.178$).

## Discussion

Our study of the southernmost breeding population of Kentish Plovers to date, found different behaviour and ecology between the temperate zone and the Al Wathba populations. The breeding season is almost continuous in Al Wathba; however, there is a peak period in April and May. The prolonged breeding season, and presumably the seasonal variation in food supply may explain why late-hatched chicks grew faster than earlier ones: this is an unexpected result, since in Turkey where breeding is limited to four months late-hatched chicks grow more slowly than early-hatched ones (Székely & Cuthill 1999).

We found four major results. First, nest survival was low, especially given that the site is surrounded by a fence that intended to exclude many terrestrial predators including foxes and stray dogs. Indeed, we found no evidence that these carnivores would take substantial numbers of eggs. A common nest predator, however, was the Grey Monitor, a native lizard in the Arabian Desert. We argue that the lizards were unusually abundant at Al Wathba for two reasons. First, Al Wathba and its breeding birds attracted predatory lizards from surrounding desert habitats, an effect we call the 'honey-pot'. Second, lizards are not persecuted inside the reserve – neither by humans nor by their natural competitors, so their populations can boom. Our data suggest that in order to preserve the balance between wetland birds and predators, it may be necessary to control Grey Monitors.

Second, survival of both nests and chicks declined over the breeding season, a common result that has been observed in Spain and Turkey (Fraga & Amat 1996, Kosztolányi et al. 2007). This is a well-known feature occurring in many temperate zone species: it may be due to increased predation rate as the season progresses, or to declining quality of birds that decide to breed.

Third, we recorded all three types of brood care (biparental, male-only, female-only) that occur in birds, although biparental care was more common than in other temperate zone populations (9.1% and 13.6% of 17+ day old broods in Hungary and France, respectively, Lessells 1984, Székely & Lessells 1993). The high frequency of biparental care suggests that an extreme environment, such as in the Arabian Desert, may favour shared brood care. Chicks are often brooded and shaded in Al Wathba and, to avoid overheating, shifting care between parents is a convenient way of increasing chick survival. Note, however, that early in the breeding season the frequency of male-only care was comparable to the frequency of female-only care: this is unusual, because in all populations that have been studied, including Snowy Plovers *Charadrius alexandrinus nivosus* (Warriner et al. 1986), male-only brood care is more common than female-only care. This suggests that in the Al Wathba population, the mating opportunities are biased toward males early in the breeding season, perhaps due to a female-biased adult sex ratio.

Finally, we found high site fidelity, as one may expect, given that Al Wathba is

surrounded by desert and nesting opportunities are limited. In spite of high site fidelity, divorce between pairs was common suggesting that site fidelity does not necessarily imply high mate fidelity. Therefore, a general relationship predicted by life-history theory between high adult survival, site fidelity and low divorce (Lloyd 2008), can be modulated by social selection. For instance, unlike many temperate and southern hemisphere plovers (Ringed Plover *Charadrius hiaticula*, White-fronted Plover *Charadrius marginatus*) Kentish Plovers do not defend territories around their nest, and this flexibility in breeding dispersal may facilitate mate change.

In conclusion, our study provided fundamental data on breeding ecology of a Kentish Plover population in the Persian Gulf. We show that features of this population (e.g. nest survival, biparental care of the eggs, and a bias toward female desertion) are similar to temperate zone populations, whereas other features (high site fidelity, high frequency of biparental care) are distinct. We speculate that some of these differences are due to extreme hot environment and/or high breeding density. Further understanding of Kentish Plover breeding ecology in Al Wathba requires continued monitoring of individually-marked plovers, and developing a new management strategy to control the number of Grey Monitors.

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**Table 1.** The fate of Kentish Plover nests in Al Wathba.

| Year | Hatched (%) | Predated (%) | Failed (%) | Unknown (%) | Total no. of nests |
|---|---|---|---|---|---|
| **2005** | 23.4 | 43.1 | 12.8 | 20.7 | 188 |
| **2006** | 19.4 | 36.4 | 14.0 | 30.2 | 129 |

**Table 2.** Home range sizes and distances between home ranges of adult Kentish Plovers in Al Wathba (Median, Lower Quartile – Upper Quartile), *n* refers to the number of adult males or females).

| | Male | Female | Mann-Whitney test between males and females |
|---|---|---|---|
| **Home ranges in 2005 (ha)** | 2.11, 0.20 – 4.85<br>*n* = 54 | 2.26, 0.59 – 6.71<br>*n* = 56 | *W* = 1354, *P* = 0.346 |
| **Home ranges in 2006 (ha)** | 3.49, 0.71 – 8.29<br>*n* = 51 | 1.83, 0.40 – 7.20<br>*n* = 49 | *W* = 1366.5, *P* = 0.422 |
| **Distance between home ranges (m)** | 245, 140 – 505<br>*n* = 77 | 330, 176 – 598<br>*n* = 70 | *W* = 2990, *P* = 0.253 |

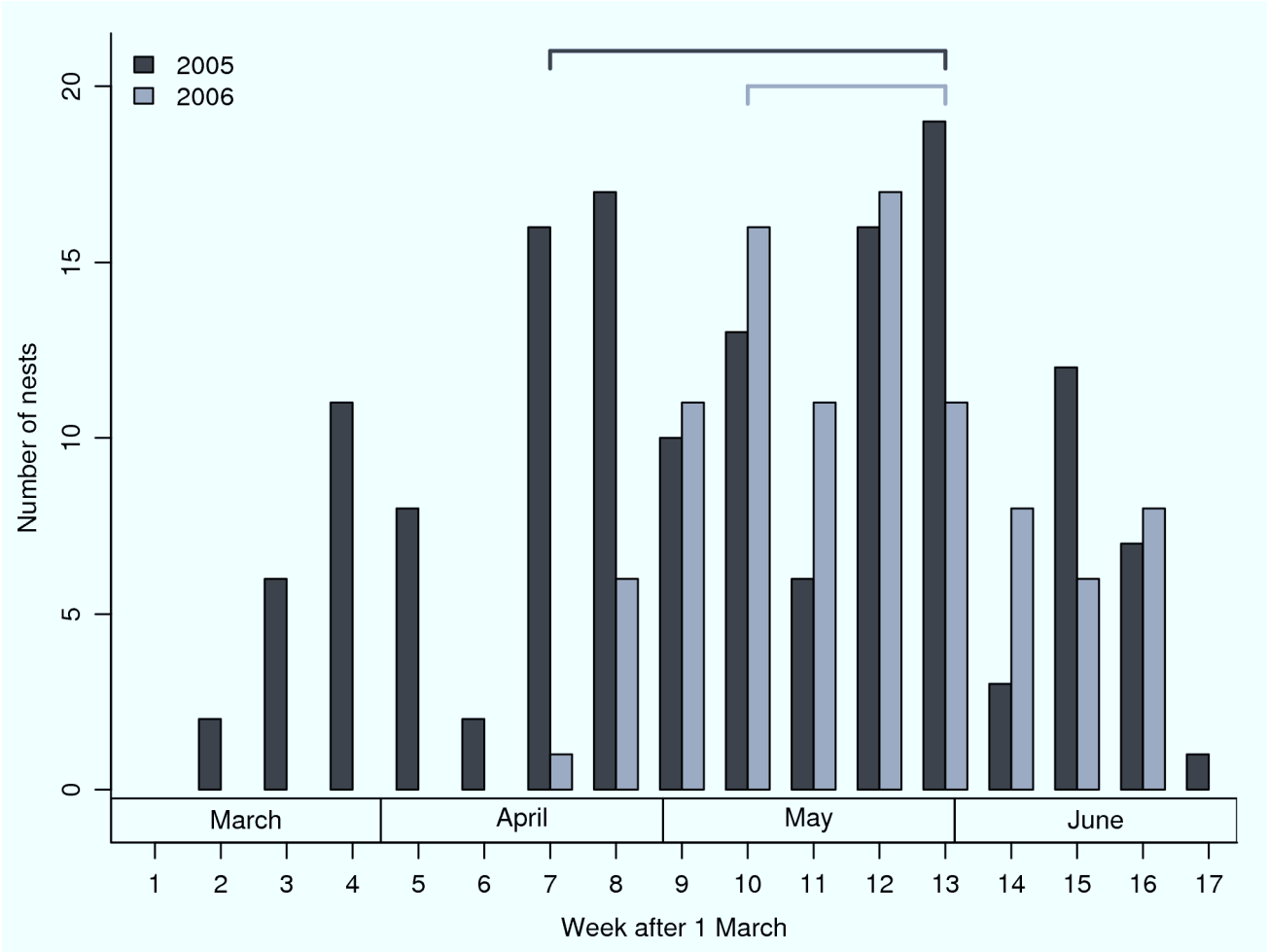


**Fig. 1.** Egg-laying season of Kentish Plover eggs at Al Wathba. The brackets over the bars represent interquartile ranges for 2005 and 2006 separately.


**Fig. 2.** Predators of Kentish Plover eggs in Al Wathba recorded on nest camera: (a) Grey Monitor *Varanus griseus*, (b) Red Fox *Vulpes vulpes*, (c) Red-wattled Lapwing *Vanellus indicus*.

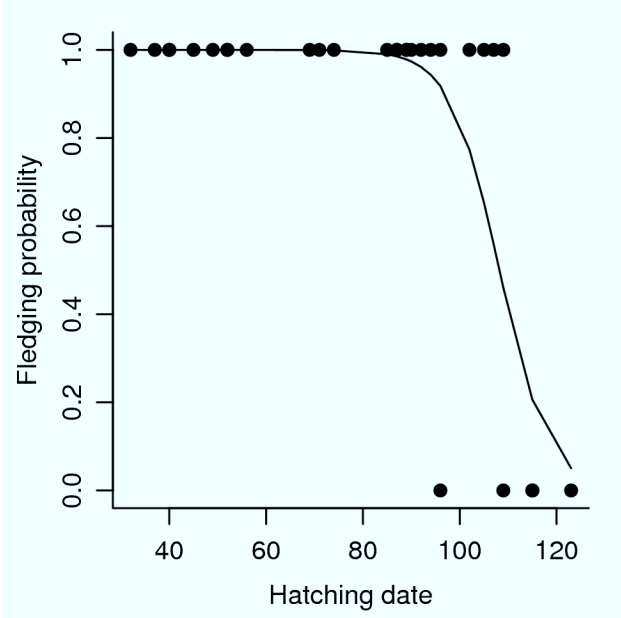


**Fig. 3.** The probability of fledging of Kentish Plover broods in relation to hatching date at Al Wathba in 2005. The line indicates fitted values of a GLM with binomial error (see text for details; hatching date is given as number of days since 1 March).

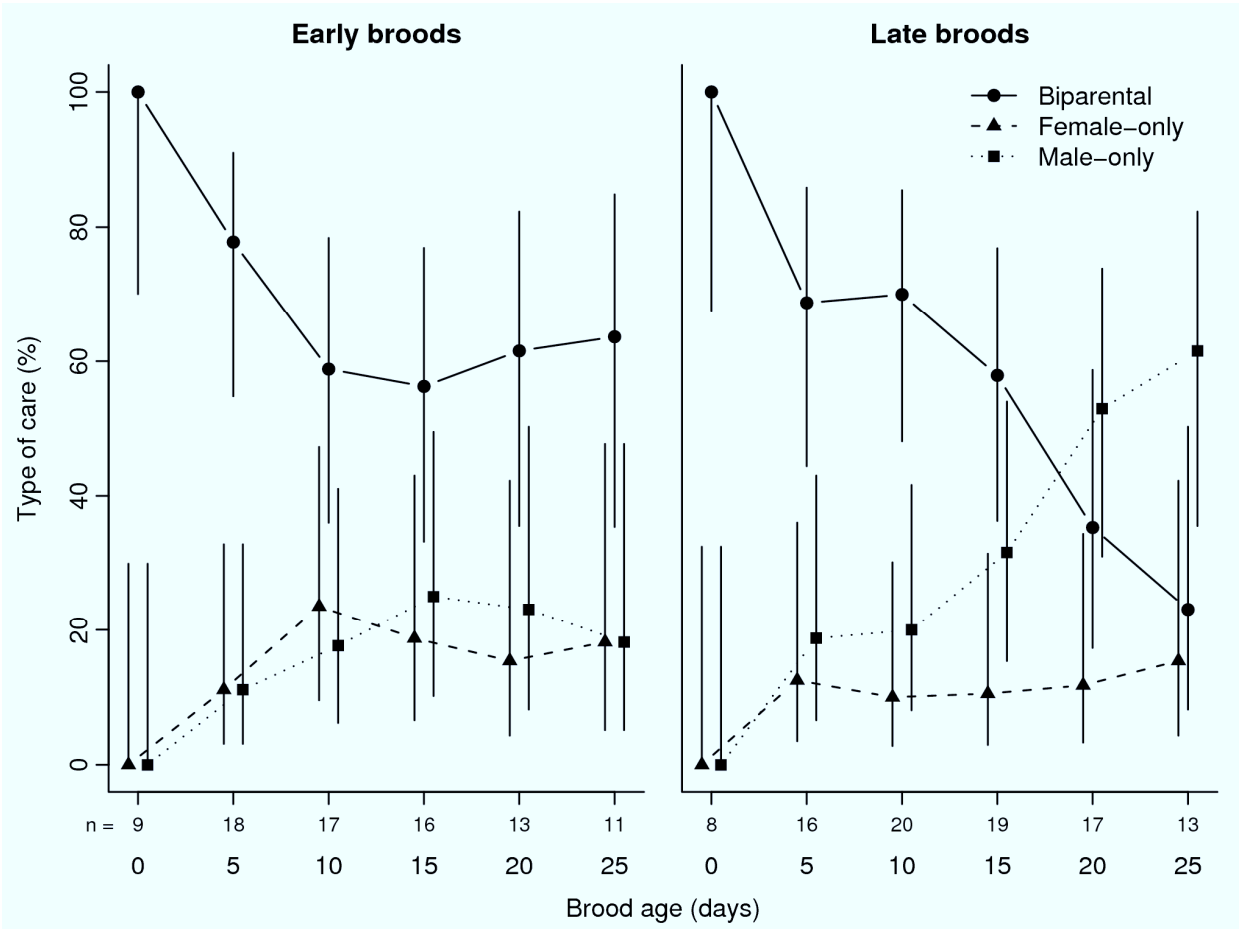


**Fig. 4.** The distribution of care types over the 0 – 25 days of brood age in Kentish Plover broods at Al Wathba in 2005. The broods were divided as early and late broods according to the median hatching date ($n$ = 27 early broods, $n$ = 26 late broods, the vertical bars represent 95% binomial confidence limits).

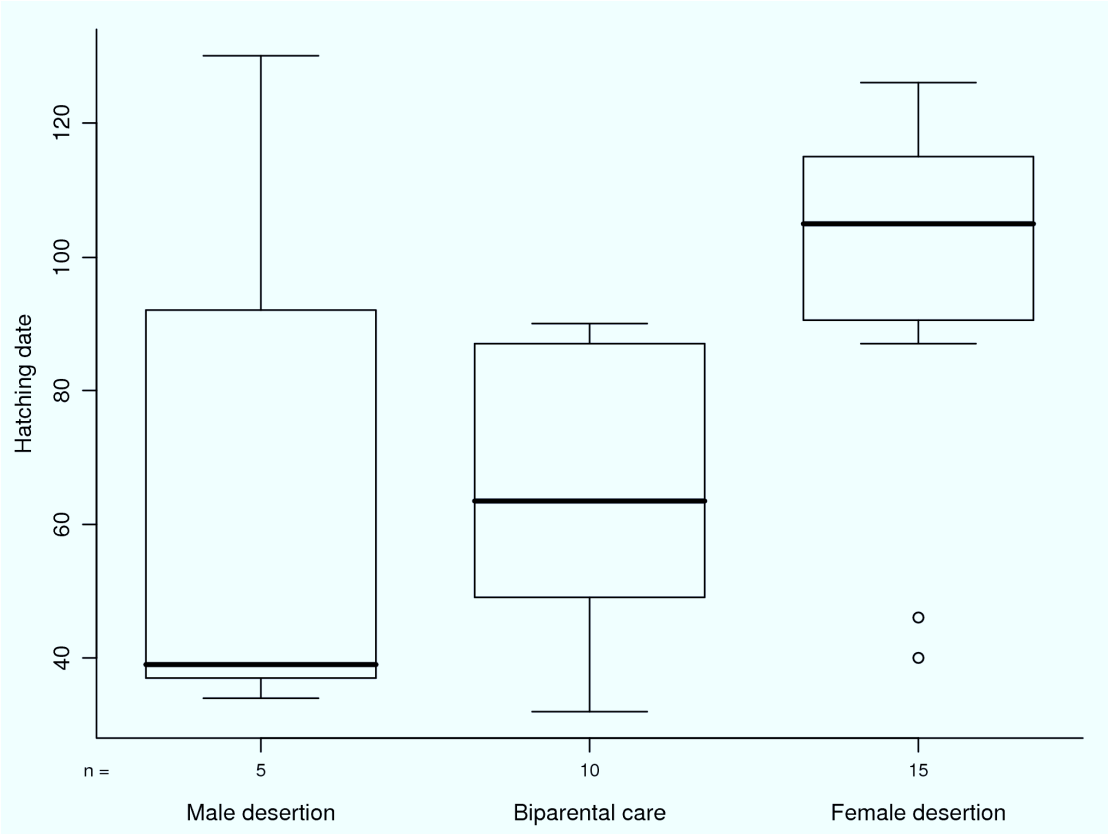


**Fig. 5.** Hatching date of broods deserted by the male, the female or neither parent at Al Wathba in 2005 (hatching date is given as number of days since 1 March).

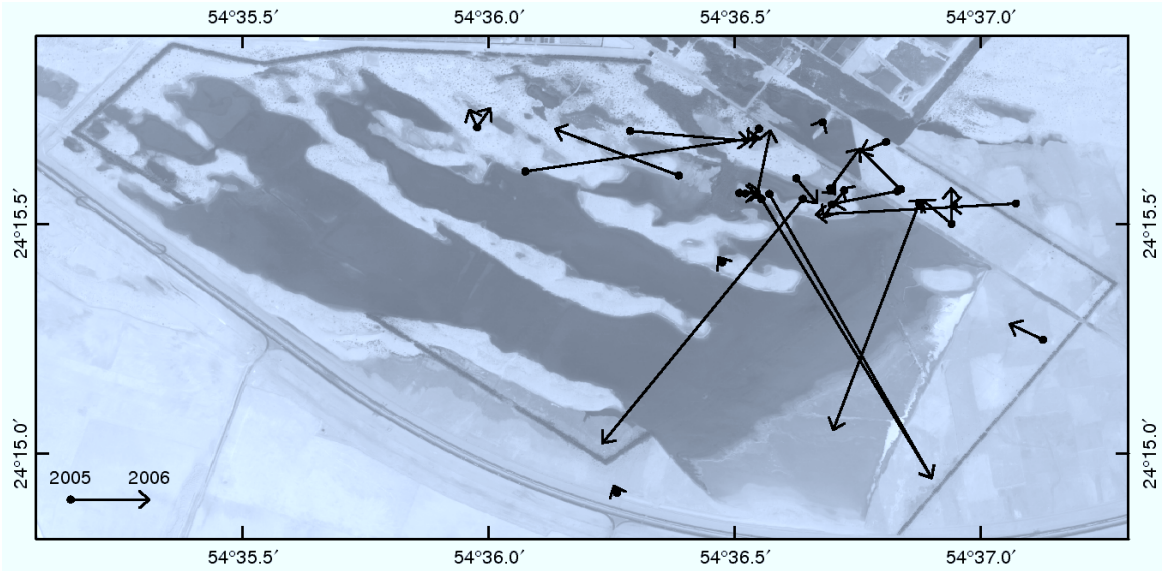


**Fig. 6.** The location of nests of individually marked Kentish Plovers that bred in both 2005 and 2006 ($n$ = 34 plovers and $n$ = 30 arrows; four pairs renested together).

Appendix II

# Parental cooperation in an extreme hot environment: incubation behaviour in Kentish plover *Charadrius alexandrinus*

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**Abstract**

Parental care often increases offspring survival, but is costly to the parents. A trade-off between the cost and benefit of care is expected so that when the offspring benefit substantially from care; parents should cooperate to rear the young. We investigated the latter hypothesis in a ground nesting shorebird, the Kentish plover *Charadrius alexandrinus* which nests in an extremely hot environment, the Arabian Desert. Midday ground temperature was often above 50°C in Abu Dhabi (United Arab Emirates), thus leaving the eggs unattended even for a few minute risks overheating and death of embryos. Through the use of video surveillance we studied incubation routines and parental cooperation by quantifying the contribution of males and females to incubation at 28 nests over 24 h. We show that ambient temperature had a significant influence on incubation behaviour, and the relationship is non-linear. Cooperation between parents was particularly strong in midday with incubation shared approximately equally. The enhanced parental cooperation was due to males increasing their nest attendance. These results suggest that ambient environment can have a major influence on care provisioning, and that when the risk of embryonic death is high the parents cooperate most strongly. Shared incubation also helps the parents cope with heat stress themselves: they can relieve each other frequently from incubation duties. Our results suggest parental cooperation is essential during incubation when the ambient environment is extremely hot. However, once the eggs have hatched the risks associated with high ambient temperature are reduced: the chicks become mobile, and they gradually develop thermoregulation. Biparental care is no longer essential and one parent may desert the family, one factor leading to the diverse breeding systems exhibited by shorebirds.

## Introduction

Parental care is beneficial for the young but often involves costs for the parents. In most bird species both parents cooperate and share care provisioning (Bennett and Owens 2002; Reynolds et al. 2002; Cockburn 2006). Biparental care is not only characterised by cooperation but also includes conflict, because it consumes time and energy, limits the opportunities for additional matings, and may put parents at risk of predation. Therefore a sexual conflict over parental care may emerge between male and female, since each parent benefits by the other working harder (Lessells 1999; Barta et al. 2002; Arnqvist and Rowe 2005; Houston et al. 2005; Székely et al. 2006).

Incubation behaviour is a major part of parental care and has implications for breeding system evolution (Ligon 1999; Deeming 2002). In precocial species which do not feed their young, incubation is a relatively large part of parental duties (Bergstrom 1986). Incubation is important to regulate the embryonic developmental temperature but it can be costly to the parents because it demands time and energy. Incubating birds spend considerable time sitting on the nest, which affects parental fitness and limits their other activities, such as foraging and attracting further mates. Division of incubation duties between parents may reduce the time and energy costs imposed upon any one of them (Deeming 2002).

Ambient temperature is one of the critical environmental variables that influences avian incubation behaviour (Conway and Martin 2000; Brown and Downs 2003; Amat and Masero 2004 a & b). The optimal temperature for embryo development in most avian species is between 36ºC and 40.5ºC (Conway and Martin 2000). If ambient temperature deviates from the optimum, parents regulate nest temperature by warming (or cooling down) the eggs (Webb 1987; Conway and Martin 2000). Cold or hot temperatures (hypo- and hyperthermia, respectively) reduce egg survival and thus may cause nest failure. However, hyperthermia is more harmful than hypothermia since hot temperatures induce embryonic mortality faster than cold ones; embryos of several species survive temporary falls of temperature down to 0ºC, whereas no embryo is known to survive above 44ºC (Webb 1987). The behaviour of incubating birds in hot environments, especially of ground-nesting birds, therefore plays a vital role in preventing eggs from overheating (Downs and Ward 1997; Brown and Downs 2003).

Ambient temperature may have adverse effect not only on the eggs, but also on the incubating birds themselves (Salzman 1982; Webb 1987; Downs and Ward 1997; Conway and Martin 2000; Amat and Masero 2004a; Amat and Masero 2007; Yasue and Dearden 2007). Extreme hot can be fatal for incubating birds, thus parents have to

balance carefully the demands of their eggs with their own risk of overheating (Grant 1982; Webb 1987; Downs and Ward 1997; Conway and Martin 2000; Amat and Masero 2004b).

Shorebirds provide excellent opportunities to investigate incubation behaviour and the effects of ambient temperature on parental behaviour. Firstly, many shorebirds nest on the ground so that their eggs are exposed to ambient environment (Grant 1982; Bergstrom 1989; Amat and Masero 2004a; Amat and Masero 2004b). Although parents may use different type and amount of nest lining, presumably to separate the eggs from ambient environment (Szentirmai and Székely 2002), their eggs are still more exposed to environmental fluctuations that in those birds that nest in tree holes or in sophisticated purpose-built arboreal nests (Hansell 2000). Secondly, shorebirds have an outstanding diversity in their breeding systems: this includes various levels of polygamy by the male or the female (or both sexes), and different levels of care provisioning by the male, female or both parents (Kam et al. 2002; Székely et al. 2006; Thomas et al. 2007). Finally, shorebirds breed in some of the most hostile environment on Earth: this includes polar breeding grounds (both Arctic and Antarctic habitats), high mountains, tropical marshes, deserts and semi-deserts (del Hoyo et al. 1996). Therefore, different adaptations are likely to be required to cope with these challenging environments, with potential implications for breeding system evolution.

We studied a small cosmopolitan ground-nesting shorebird, the Kentish plover, *Charadrius alexandrinus* (body mass approximately 42 g), which breeds in temperate and subtropical environments where ambient temperatures commonly reach over 50ºC during daytime (Purdue 1976; Grant 1982; Amat and Masero 2004a). Nests are sparsely filled with material such as straw, pebbles, mollusc shells and algae which may act as insulation materials to help to regulate the egg temperature (Purdue 1976; Szentirmai and Székely 2002). Both parents participate in incubation: females usually incubate in the daytime whereas males incubate during night (Fraga and Amat 1996; Kosztolányi and Székely 2002).

The Kentish plover is an ideal model for studying parental behaviour, since it has variable parental care both within and between populations. Both parents incubate the eggs although after the eggs hatch one parent (the male or the female) deserts the family and seeks a new mate; so that monogamy, polygyny and polyandry may all occur within a single population (Warriner et al. 1986; Lessells 1984; Amat et al. 1999; Székely and Cuthill 2000; Székely et al. 2006). The transition from shared incubation to uniparental care is an excellent paradigm to understand how and why animals may shift

from cooperation that enhances to both parents' interest to desertion that is only beneficial for the deserting individual but costly to the deserted parent.

Here we investigate parental cooperation during incubation by quantifying incubation by males and females in Kentish plovers nesting in the Arabian Peninsula where ground temperatures may exceed 60°C at midday during the breeding season. The objectives of our study were to answer two questions: i) Is the behaviour of the male or the female influenced by ambient temperature? ii) Does ambient temperature influence parental cooperation during incubation?

## Materials and methods

### Study area

Fieldwork was carried out in Al Wathba Wetland Reserve between 13th of March and 23rd of July 2005, and between 26th of April and 12th of July 2006. This reserve is located approximately 40km south-east of Abu Dhabi in the United Arab Emirates (24° 15.5' N, 54° 36.2' E). The fenced reserve with a total size of about 465 ha is composed of artificially created water bodies that are surrounded by sand dunes. About 200 pairs of Kentish plover breed within and around the reserve.

### Data collection

Kentish plovers are sexually dimorphic during the breeding season which allows identification of sexes from photos (Cramp and Simmons 1983); adult males have black eye-stripes; black frontal head bars and incomplete black breast-bands, whereas these areas are pale brown in adult females. Sexual dimorphism in plumage characteristics between sexes decreases over the season, therefore at four nests in 2005 the eye and head stripe of males were dyed using black permanent marker to enable discrimination between males and females from the nest photos (see below).

Activities at the nests were recorded using a small spy camera (Outdoorcam, Swann Communications Pty. Ltd., Richmond, Victoria, Australia) positioned about 1m from the nest. The camera was connected to a digital video recorder (MemoCam, Video Domain Technologies Ltd., Petah Tikva, Israel) that recorded an image every 20s. The camera was equipped with infrared lights that allowed capturing the images of incubating plovers during night. Power was supplied by a car battery. The ambient temperature was measured by a thermo-probe which was placed about 25 cm from the nest scrape at ground level. The probe was connected to a data logger (Tinytag, Gemini Data Loggers Ltd., Chichester, UK) that recorded the temperature every 20s. Ground

temperature often exceeded 50 °C at midday (Figure 1); the maximum ground temperature we recorded was 64.8°C.

All parts of the system (except the camera), and the cables were hidden underground to minimize the disturbance to the birds. Date of egg-laying was known for clutches, or was estimated by floating the eggs in lukewarm water (Székely et al. 2008). Egg-laying is given as the number of days since 1 March.

Ambivalent identification of male and female, and records when the parents were disturbed (e.g. during daily function tests of the data recording systems) were omitted from analyses (< 2.3 % of all records). At 20 nests egg temperatures were experimentally manipulated for a separate study, thus for the latter nests only data from control period that preceded the experimental manipulation were included in the current analyses. In total, data from 28 nests (20 and 8 nests from 2005 and 2006, respectively) were included in the analyses.

**Statistical procedures**

24 h recording starting at midnight (0.00 h) was taken for each nest, and considered as the unit of analysis. Each day was divided into twelve 2 h time periods. Three behavioural variables were calculated for each interval: (1) *total incubation*, the percentage of time when the eggs were incubated by either parent; (2) *male incubation*, the percentage of time when the eggs were incubated by the male, (3) *female incubation*, the percentage of time when the clutch was incubated by the female. These variables (converted to proportions) were arcsine square-root transformed. The average ground temperature measured during each period was taken as the ambient temperature.

Estimates for repeatability of behaviour give a standard measure of individual consistency and may be treated as a measure of susceptibility of a particular behaviour to environmental influences (Boake 1989; Nakagawa et al. 2007). Following Harper (1994) we calculated repeatability of the incubation behaviour for 8 nests where data for two full days were available. We used repeatability (R) for interpretation rather than statistical significance, because the latter is influenced by sample size (Nakagawa & Cuthill 2007).

The influence of ambient temperature on incubation behaviour was investigated using linear mixed-effects models (lme, Pinheiro and Bates 2000) with nest identity as a grouping structure since parental behaviour is not independent between two-hour time periods for a given nest. Year, egg laying date and the number of days since the start of incubation potentially influence incubation behaviour. We carefully tested all three

variables on our response traits (% total incubation, % male incubation, % female incubation), however, none of them had a significant effect (mixed-effects models, $P \geq 0.060$ in all cases) therefore these variables were not analysed further.

The initial model of total incubation included time period (fixed factor), ambient temperature (second degree orthogonal polynomial covariate) and their interaction because Conway & Martin (2000) found that avian incubation behaviour and ambient temperature are not linearly associated. The initial model for male and female incubation included time period (fixed factor), ambient temperature (second degree orthogonal polynomial covariate), the incubation by the other sex (fixed factor) and all second-order interactions. All models included a random intercept term for each nest. The initial models were fitted using maximum likelihood method, and model selection was carried out using AIC. The final models were refitted using restricted maximum likelihood.

To illustrate the results of the final mixed-effects models (Table 1) on Figures 3-5, we fitted mixed-effects models containing the variables showed on the figures and present the back-transformed fitted values and the observed data. We used R version 2.7.1. for statistical analyses. Values are given as mean ± SE unless stated otherwise.

## Results

### Repeatability

Total incubation was moderately repeatable (one-way ANOVA, R = 0.54, $F_{7,8} = 3.428$, P = 0.053). Male incubation showed low repeatability (R = 0.27, $F_{7,8} = 1.760$, P = 0.223), whereas female incubation was not repeatable (R = -0.01, $F_{7,8} = 0.992$, P = 0.498).

### Daily routine

Overall, the mean incubation coverage was 85.7 ± 1.1% over the full day (n = 28 nests). Females attended the nest 44.5 ±1.7% of their time, whereas males attended the nest 41.3 ± 1.6% of their time. At night (20:00 - 6:00h) total incubation was 90.8 ± 1.4%, with females and males spending 33.2 ± 3.6% and 57.6 ± 3.6%, respectively. In contrast, during daytime (6:00-20:00h) nests were attended 82.4 ± 1.4% of the time, with females and males attending 52.1 ± 2.1% and 30.4 ± 1.8% respectively.

The nest was attended by either parent over 70% of time in each period except 18-20 h, and the highest attendance was in midday (Figure 1). Male and female

incubation routines were different: females incubated the eggs mostly in morning and males in the evening and at night (Figure 1).

**The influence of ambient temperature on parental behaviour**

Ambient temperature influenced incubation and the effects were often not linear (Figures 2-4, Table 1). In the morning as ambient temperature increases, total incubation decreased with temperature. In midday, however, total incubation increased with temperature (Figure 2). In late evening, incubation decreased again with temperature.

Males and females responded differentially to ambient temperature during different parts of the day, as indicated by the highly significant interaction terms between time period and temperature (Table 1). During midday males usually increased incubation with temperature, whereas females decreased (Figures 2 and 3).

**The influence of ambient temperature on parental cooperation**

Female and male incubation tend to show a trade-off, although the strength of this relationship varies significantly over the day (Table 1, Figure 5). In morning (6-10 h) and in the evening (16-20 h) the inverse relationship between male and female incubation is poor. In contrast, during midday (10-14h) the parents practically cover the nest continuously.

**Discussion**

The repeatability estimates indicate that, for a given nest, total and, to a lesser degree, male incubation are consistent whereas female incubation is not consistent. Although our results are based on a small sample size (n=8), the trend is interesting in the context of sexual selection. The strength of selection process partially depends on the consistency of the trait. For example, females may prefer males when male parental care is at least to some degree consistent across breeding seasons. Consistency in male care but not in female care has also been reported in house sparrow *Passer domesticus* in which feeding rates of males, but not females, were highly repeatable across years (Nakagawa et al. 2007). Similar to Kentish plovers, the repeatability of incubation effort was low to moderate in house sparrows.

Our study provided two further major results. Firstly, ambient temperature had a significant influence on incubation behaviour, and the relationship was non-linear. Incubation behaviour decreased with ambient temperatures until reaching the (presumed) optimal temperature for embryo development, and increased when ambient

temperature exceeded the optimal egg temperature. Secondly, the increase in ambient temperature has important implications for parental cooperation during incubation, whereby Kentish plover parents showed far tighter coupling in their incubation behaviour when the daytime ambient temperature was high. The tightly mirrored responses appeared in midday when parents maximised total incubation whilst simultaneously minimising their own contribution (and heat load), reaching an apparently stable overall share of 50% each.

In agreement with the results of our study, Purdue (1976) reported that attendance at snowy plover *Charadrius alexandrinus nivosus* nests increased during hot parts of the day. During these times the intervals between shifts are diminished, whereas in late afternoon the attendance of parents decreased, possibly because the latter period was suitable for peak foraging activities and ambient temperature do not harm the embryo. Amat and Masero (2004a) suggested that hot ambient temperature may limit incubation bouts, since females may be incapable of incubating the clutch for long periods at extreme temperatures. Hence the males' proportional increase in diurnal incubation can lead to short incubation bouts by females in midday when parental care is most difficult. The responses of plover parents, however, appear to be different between populations, since in Germany, close to the northernmost edge of their breeding distributions, female Kentish plovers incubated over 80% whereas males incubated less than 20% during 10-18h in a temperate environment where temperatures are below 35 °C (Rittinghaus 1961).

An alternative explanation for the tight division of incubation is coercion: the female coerces the male to work harder. It would need experimental test to separate whether the male or female drives the tighter pattern although we argue that female coercion is unlikely, because the body sizes of males and females are similar and both sexes are equipped to fight (Kosztolányi et al. 2006, see further arguments above).

Results of this study may be important for two reasons. Firstly, they suggest that incubation may put adults under severe heat stress in ground-nesting birds in a desert environment (see also Amat and Masero 2004a). However, interrupting incubation for more than a few minutes would be lethal for the embryos (Amat and Masero 2004a). These conditions should favour tight coupling of parental behaviour, and the overt cooperation observed; the adults thereby reduce risk to themselves and their eggs. Males may be forced to participate in daytime incubation because the female can not manage the task alone and otherwise the embryo would die. Thus, extreme temperatures may

increase the level of cooperation and reduce their sexual conflict over care (Amat and Masero 2004a).

Secondly, our results imply that once the eggs have hatched the risk factors associated with high ambient temperature are reduced; the chicks become mobile, adults and young have a suit of new behaviours to thermoregulate such as bathing or move under shades. Since the chicks are more independent, desertion of one of the parents may become less costly. Biparental care with high parental cooperation may still be favoured by other environmental variables such as localised food distribution and high predation on the chicks. On the one hand, Kosztolányi et al. (2006) found that when food distribution was patchy, the density of plovers and competition between plover families increased. Parents spent more time defending their young and biparental brood care lasted for longer when food was abundant but localised. On the other hand, Fraga and Amat (1996) found that biparental care was longer in an inland saline lake with heavy predation by gull-billed terns *Sterna nilotica* than in other populations.

Our results display the behaviour captured by real-time negotiation models (McNamara et al. 1999) which propose that parents negotiate their effort by responding to each other during a given breeding period. Several lines of evidences support these arguments. First, females desert more often than do males and the desertion normally occurs after hatching (Warriner et al 1986; Székely and Williams 1995; Székely and Cuthill 2000; Kosztolányi et al. 2003; Amat et al. 2000). The desertion is more likely when environmental conditions are good because one parent will be enough to look after the chicks (Kosztolányi et al. 2006; Fraga and Amat 1996). Second, females mainly incubate through the daylight hours, whereas males incubate during the night (Fraga and Amat 1996; Kosztolányi & Székely 2002), however if the workload becomes unbearable for one parent alone, as shown in this study during the hot hours, the male will assist in incubation. It is not yet known what may drive the diurnal versus night-time shift between sexes, although it seems different shorebird species exhibit different daily routines. Third, the participation of male in diurnal incubation in hot environments is greater in exposed sites than in covered ones (Amat and Masero 2004b).

In conclusion, this study suggests hot environment favours cooperation between Kentish plover parents during incubation. The increased parental cooperation is probably essential, since a single parent cannot protect the eggs and/or itself from overheating. If cooperation is enhanced by high ambient temperature, with changes in global climate we may see implications on breeding systems in near future.

Experimental analyses of male-female interaction during incubation, and comparing the incubation responses of males and females across different plover populations appear to be viable options to reveal the relationship between ambient environment and parental cooperation.

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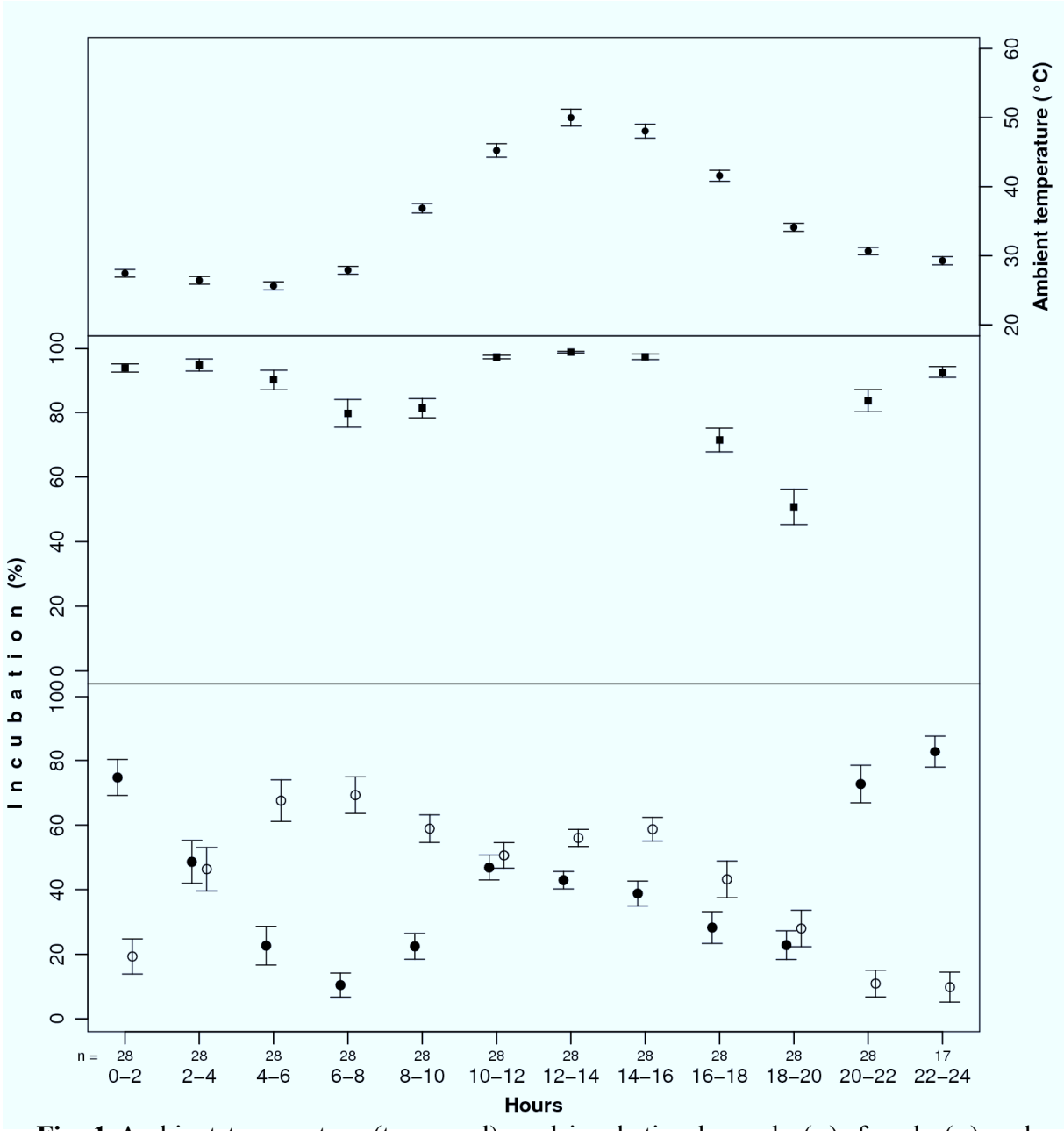


**Fig. 1** Ambient temperature (top panel), and incubation by male (●), female (○) and both parents (■) at Kentish plover nests in Abu Dhabi (mean ± SE for each two-hour time period, n is the number of nests used in the analyses).

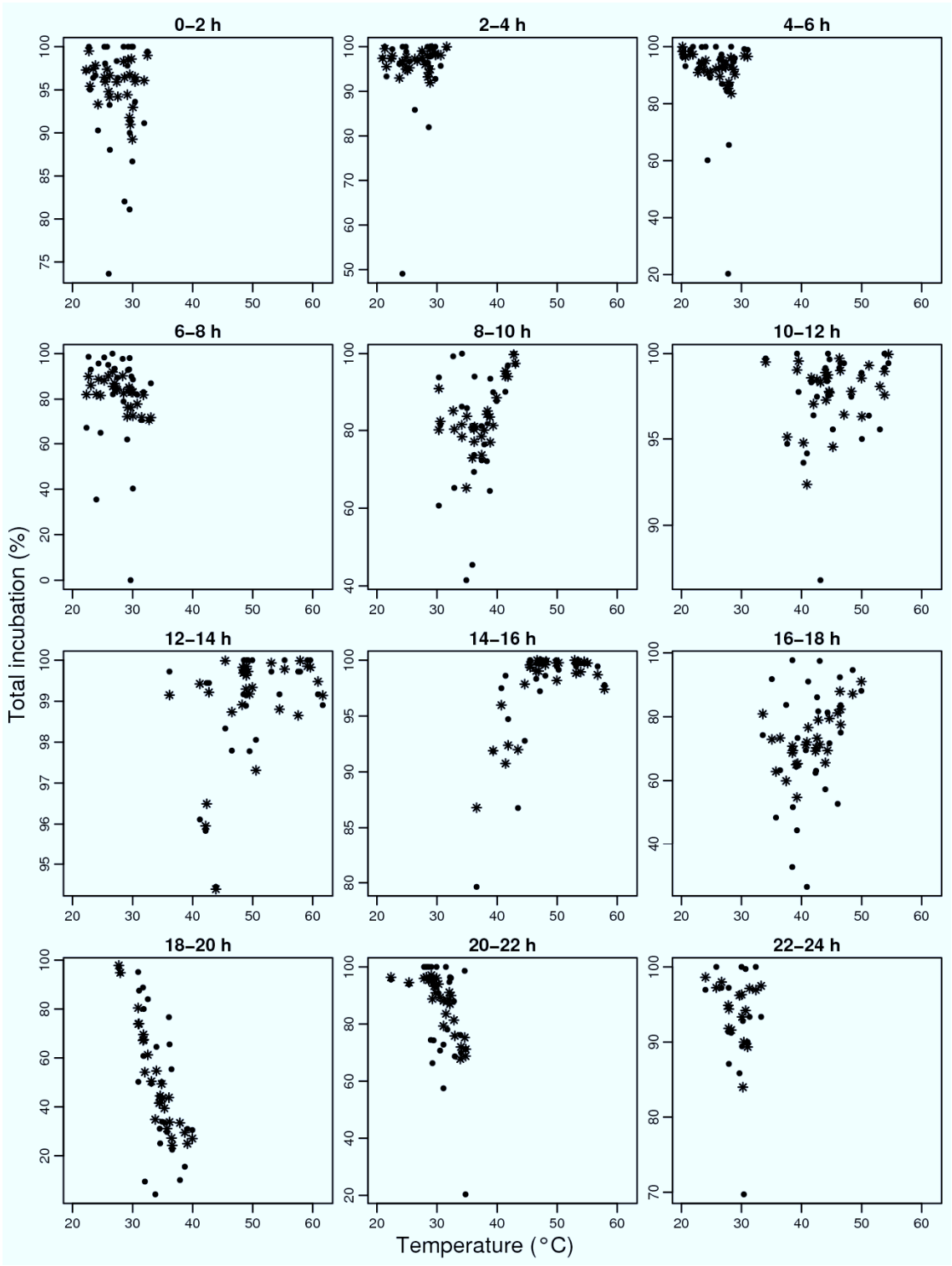


**Fig. 2** Total incubation (%) in relation to ambient temperature (°C) in two-hour periods: observed and fitted values (*) are shown from mixed-effects model (see Methods for details).

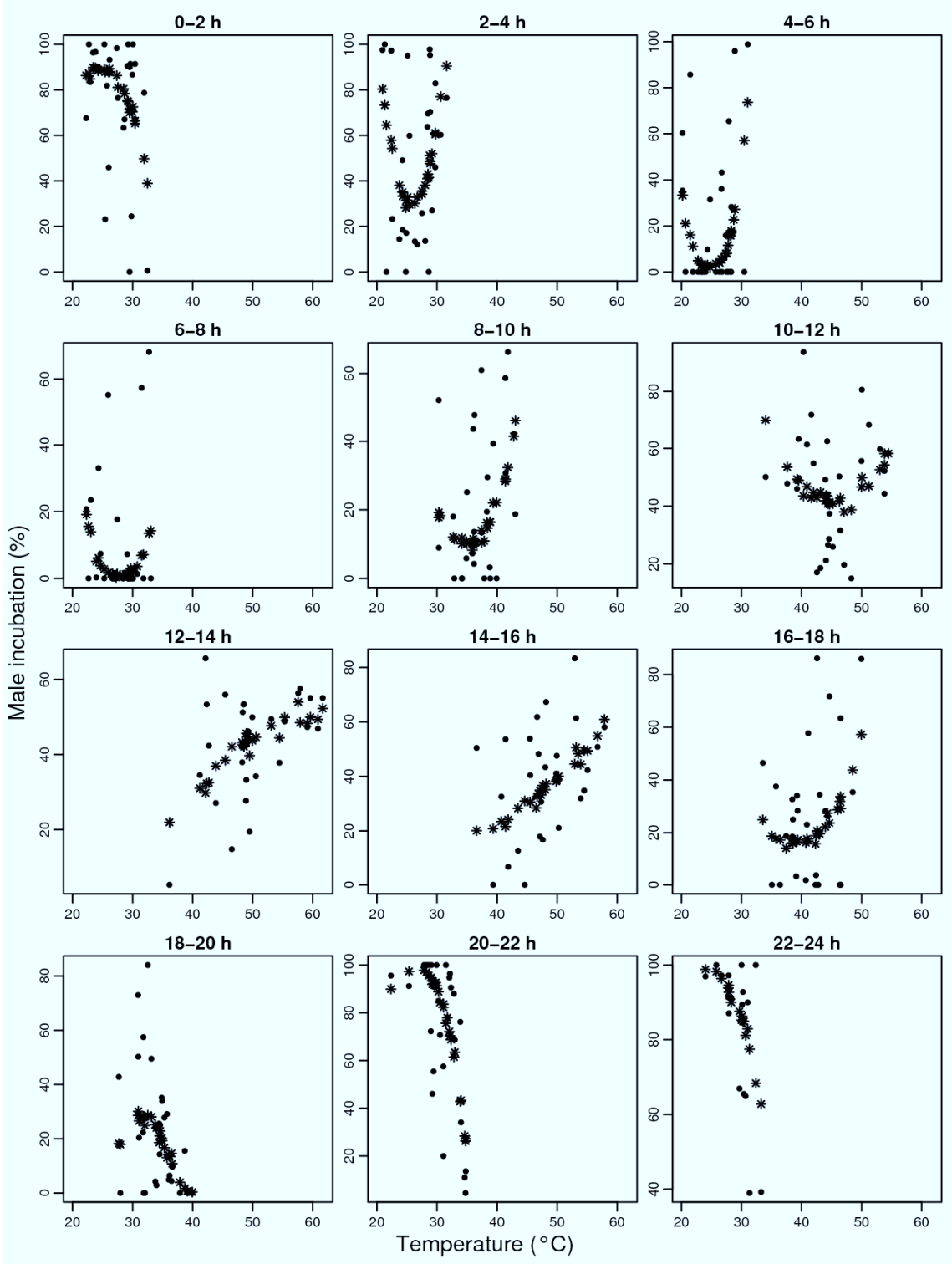


**Fig. 3** Male incubation (%) in relation to ambient temperature (°C) in two-hour periods: observed and fitted values (*) are shown from mixed-effects model (see Methods for details).

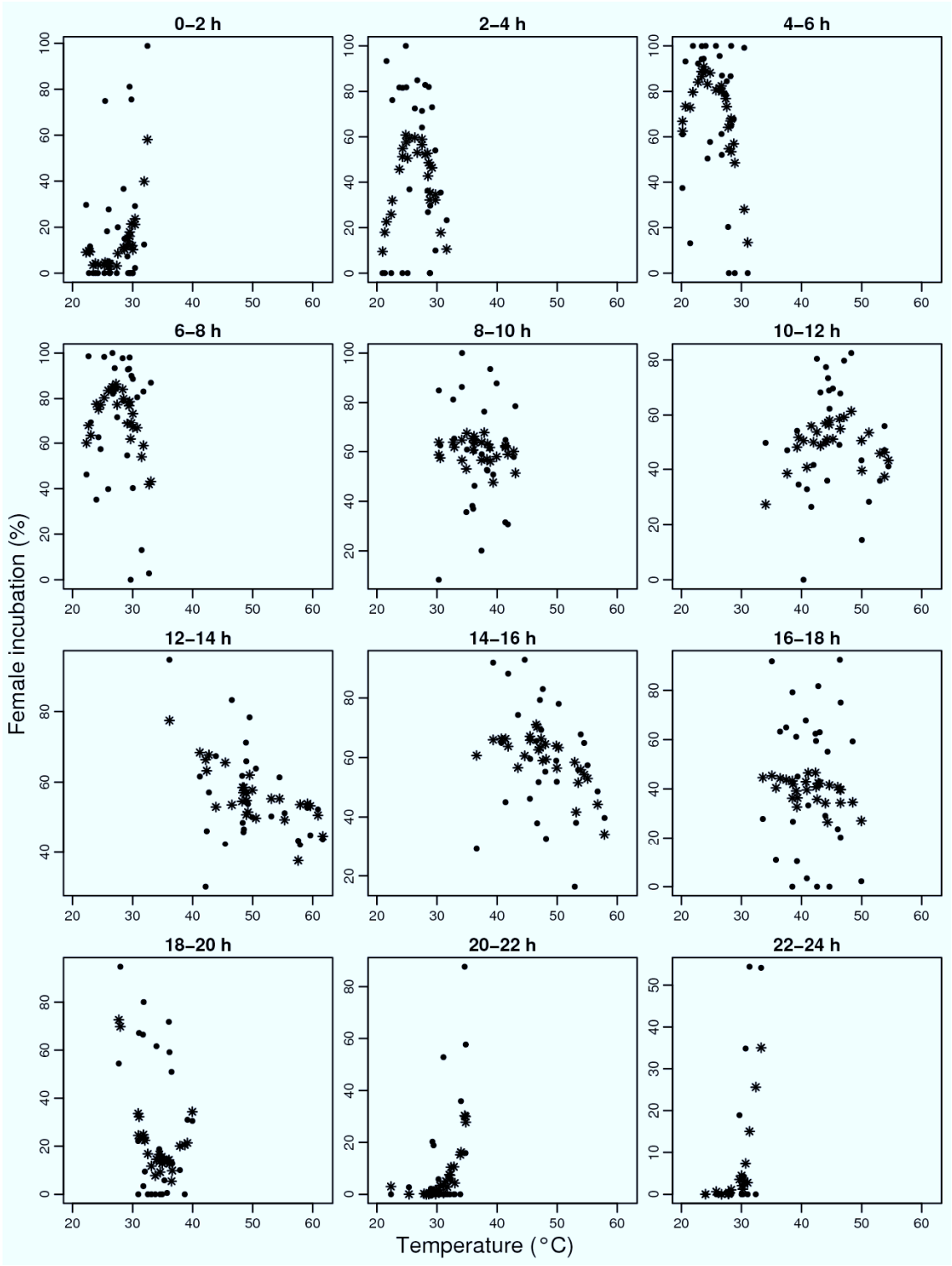


**Fig. 4** Female incubation (%) in relation to ambient temperature (°C) in two-hour periods: observed and fitted values (*) are shown from mixed-effects model (see Methods for details).

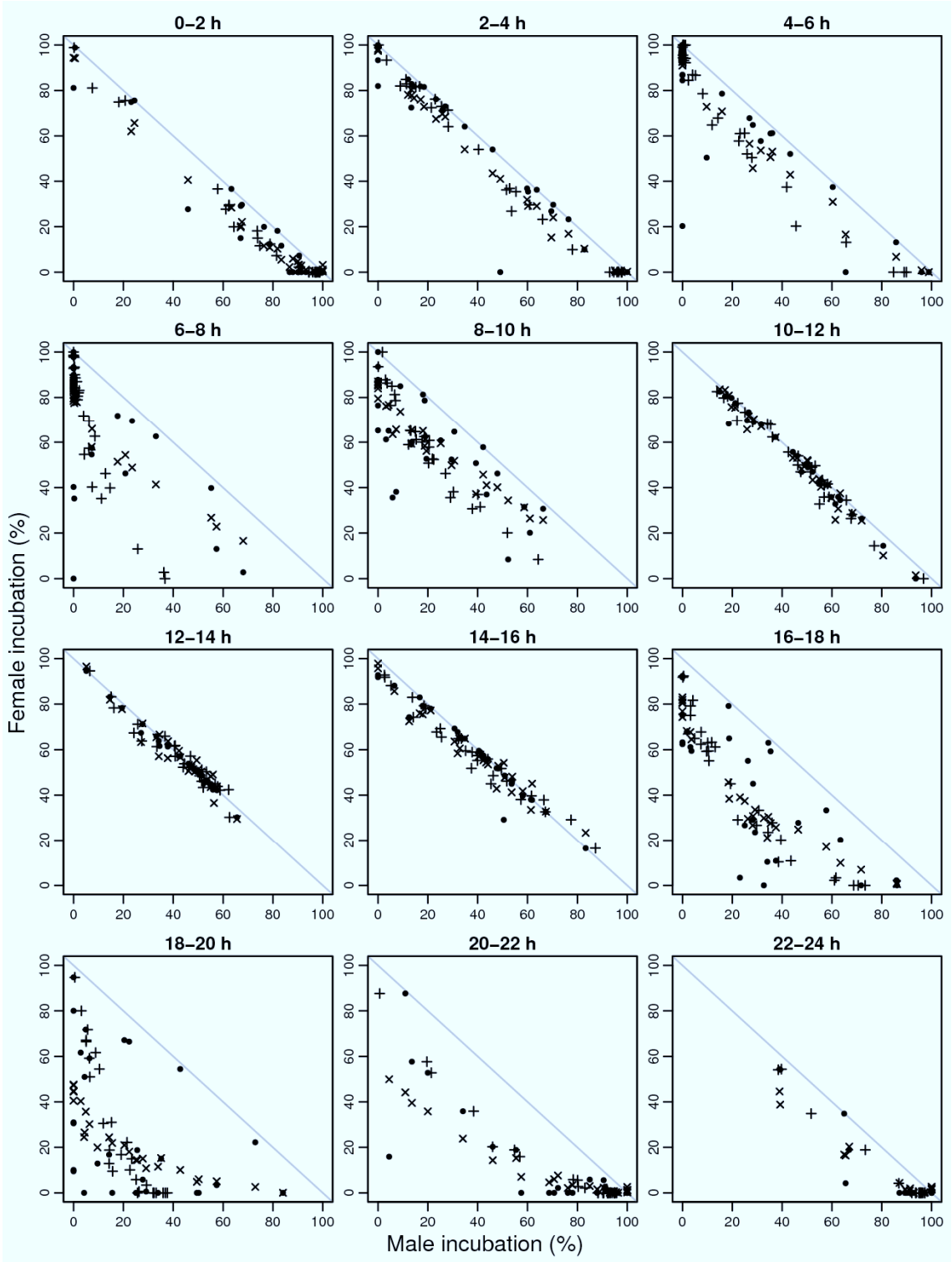


**Fig. 5** Female incubation (%) and male incubation (%) in two-hour periods: observed and fitted values (× female incubation as response, + male incubation as response) from mixed-effects model (see Methods for details). The grey line (-1 slope) shows the 100% total incubation threshold. Each dot represents a nest.

**Table 1** Final mixed-effects models of incubation behaviour in the Kentish plover (Type III SS ANOVA). Ambient temperature was included in the models as second degree orthogonal polynomial. Statistically significant terms are in italics.

.

| **Explanatory variables** | **Response variables** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **% total incubation** $df_{error} = 262$ | | | **% male incubation** $df_{error} = 250$ | | | **% female incubation** $df_{error} = 261$ | | |
| | **df** | **F** | **P** | **df** | **F** | **P** | **df** | **F** | **P** |
| Time period | 11 | 1.44 | 0.157 | *11* | *2.61* | *0.004* | 11 | 0.81 | 0.632 |
| Ambient temperature | 2 | 0.25 | 0.782 | 2 | 0.35 | 0.704 | 2 | 0.75 | 0.475 |
| Female incubation | | | | *1* | *118.21* | *<0.001* | | | |
| Male incubation | | | | | | | *1* | *614.79* | *<0.001* |
| Time period x ambient temperature | *22* | *3.25* | *<0.001* | *22* | *2.95* | *<0.001* | *22* | *2.51* | *<0.001* |
| Time period x female incubation | | | | *11* | *3.36* | *<0.001* | | | |

See Materials and methods for details of the models

# Appendix III

## Sexual conflict over parental care: a case study of shorebirds

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Appendix IV

# Practical guide for investigating breeding ecology of Kentish plover *Charadrius alexandrinus*

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# Practical guide for investigating breeding ecology of Kentish plover *Charadrius alexandrinus*

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*Version 3*, 1 April 2008

*Photos by T. Székely, A. Kosztolányi & C. Küpper*

# Rationale

The Kentish/snowy plover *Charadrius alexandrinus* is a small cosmopolitan shorebird (body mass about 40-44 g). In the last few years we have developed a suite of methods to investigate its behaviour and ecology in the field. We thought this practical guide may be useful for students and researchers with an interest in small plovers. Some aspects of these methods may be relevant for other shorebirds and ground-nesting birds in general.

Our fundamental motivation in writing this guide is to show that the Kentish plover is an easy species to work with, if one is willing to pay attention to a few potential pitfalls. We hope that this guide will elicit further research. Please contact us if you have questions and comments, and let us know of any errors. Note that Kentish plovers have been studied in several countries and by a good range of researchers, and we don't claim that our methods work best.

Many Kentish plover populations are now declining. You need to be sensible about fieldwork, and carefully evaluate the costs and benefits of using a particular method. The last thing you want is to put an extra burden on plover populations - they have a hard time anyway to cope with predators, floods and threats humans are imposing upon them.

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# Essential breeding ecology

Good reviews of Kentish/snowy plover (KP henceforward) natural history can be found elsewhere (Cramp & Simmons 1983, Page *et al.* 1995, Amat 2003), and here we only focus on essential aspects. KPs are migratory in most parts of their range, although populations close to the equator are only partially migrant or resident. They breed on edges of saline lakes and lagoons, and inhabit salt-marshes and sand dunes. Their breeding season usually lasts for about 2-5 months; populations in the north tend to be single-brooded, whereas southern breeders may double (or triple) brood. Failed breeders often re-nest.

Adult males and females have dimorphic plumage. Males have incomplete black breast-bands, black eye-stripes and a black frontal head bar, whereas these areas are pale brown in females. Males also sport a cinnamon nape and crown, although there is a substantial plumage variation among breeding populations. In early breeding season the sexes are easy to distinguish, whereas the difference in plumage between sexes becomes blurred as the season progresses.

Kentish plovers lay their eggs in a small depression on the ground scraped by the male. The modal clutch size is three eggs, and the eggs hatch after 25-26 days of incubation. Both sexes incubate the eggs; females incubate mostly during the day, and males incubate mostly during the night. The parents lead the chicks away from the nest-scrape within a few hours of hatching. The parents attend, brood and defend their chicks for about 4-5 weeks, but they don't provide food for them. One parent (the male or the female) often deserts the brood, and re-nests with a new mate. Thus many KPs are socially monogamous, although both polygyny and polyandry occur in most populations that have been studied to date.

## Searching for nests

Equipment needed: binoculars, spotting scope, mobile hide (see ***Appendix 1***) or car.

There are three main methods of nest search:
(i) On foot. Potential breeding sites can be screened by walking and searching for nests. Empty nest scrapes (with or without some nest materials) often indicate the presence of active males. On sand dunes, the plover footprints tend to concentrate around their nests. On salt marsh, it is worth looking for sites that are somehow more elevated from the rest, so that the nest is less prone to flooding. Objects that break monotony of the ground (e.g. debris, deep footprints, drift-wood) are often preferred locations. Eggs in fresh (or incomplete) nests tend to be fully exposed, and as incubation progresses nest materials gradually accumulate so that the eggs may be nearly completely covered.

(ii) Spotting incubating parents. The observer should sit on an elevated vantage point (such as a dyke or on a sand bank), or inside a car or mobile hide. Incubated plovers can be spotted by their distinctive white breast, or their contour against the background. Sitting plovers can be easily distinguished from incubating ones: the incubating parents appear to have bulging breasts, and when they run off the nest they often throw nest materials towards it. The behaviour of non-incubating plovers is more relaxed; they often preen and alternate between sitting and standing, and they don't have the typical shaking moves of incubating parent when settle on the eggs.

When using a mobile hide (***Appendix 1***) it is a good idea to move for a stretch of 40-50 m, and then stop and look around using both binoculars and spotting scopes. Make sure you screen the same spot from different positions – you may be surprised how many nests you miss by superficially looking around. It is worth checking the area close to the hide, because some plovers continue incubating until the hide is only a few meters from them. Make sure you screen your path very carefully to avoid pushing the hide over a nest.

Make notes about males that are advertising and defending a particular spot, and pairs that are profusely display, scrape and copulate. A few days later you may find their nest – although this is not guaranteed since many unmated males and pairs eventually nest elsewhere.

(iii) Watching parent(s) returning to the nest. Plovers can be very tolerant of observers inside a car or hide, but they are wary of observers on foot. The flushed parents may run back to the nest in a straight line, although cautious individuals may zigzag, or exhibit seemingly foraging movements whilst gradually approaching their nest. Carefully note the location where the plover disappeared from your sight: often this is a telltale of a nest.

Beginners often confuse false-brooding (a form of nest defence) from real incubation; the former is usually exhibited to observers on foot, and they can be displayed in unsuitable habitats (e.g. in shallow water or wet mud). If you spot a nest from a distance, you need to walk to the nest in a straight line whilst fixing your eyes on the suspected nest location. Many nests are well camouflaged, and a mistake of 1 m may mean you can't see the eggs even though they are right in front of you. Watch your steps near nests and ignore the displaying parents: the last thing you want is crushing the eggs.

If you flush several plovers, it is a good practice to choose the most anxious plover (the

one that does lots of head-bobbing and short abrupt runs), and then follow his/her movements for 10-15 minutes. Bear in mind that if a plover is very agitated, for instance it zigzags for 5-10 minutes in front of you or tries to lure you away; these often mean you are too close to their nest (or chicks). You need to carefully reverse and let the parents return to their nest (or brood).

***Carry out nest search sensibly, especially if the weather is very cold or hot:*** by keeping the parents away from their nest, you may fatally expose the eggs. A good practice is to work swiftly and efficiently in a location, and move to a different location as soon as possible to let plovers resume their normal life. ***It may take years to figure out and avoid* 'the elephant in a china shop'** ***effect, but the sooner you start realising your potential effects the better.***

## Measuring and checking nests

Equipment needed: Nest notebook, GPS device, sliding calliper, small jar filled with fresh water.

Once a nest is located, you need to record the essential data (***Appendix 2***). Work efficiently and don't spend more than 5-10 min at the nest: this is NOT the place to celebrate, or discuss the latest gossips. Try to leave as few footprints as possible, and don't approach the nest if visual predators (e.g. rooks, shrikes) or humans are around. If you suspect that nocturnal predators (e.g. foxes, hedgehogs, jackals) may locate the nest using your scent, you should avoid approaching nests and handling eggs in late afternoons and in the evening.

We note the nest and egg number on the blunt end of egg using black permanent marker, e.g. 34/2. These numbers are helpful when you only find egg shell remains. People often worry about the harmful effect of marker solvent on embryonic development: we have not seen any evidence of this.

You need to measure egg length and breadth using a sliding calliper (***Figure 1***). Hold the egg horizontally in your palm, and then gently push the sliding calliper downwards and simultaneously pull apart the jaws of the calliper. Record the measurement when the egg squeezes through between the jaws for the first time. Do NOT force the calliper: the eggshell is thin, and can easily break.

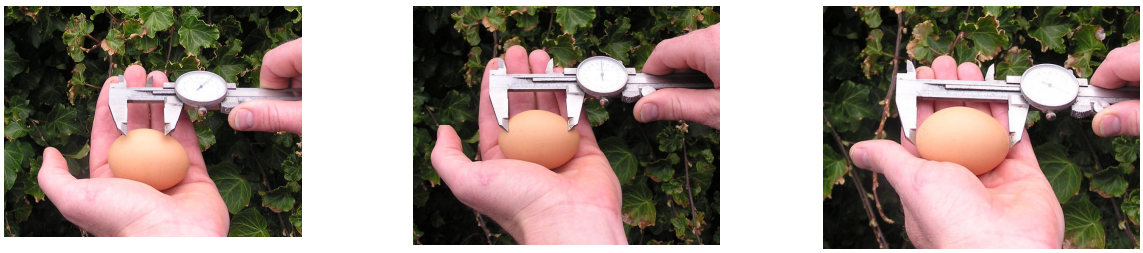


***Figure 1****. Process of measuring eggs (from left to right) as illustrated with a hen egg.*

To get accurate measurements, you may repeat the process three times and take the median of readings. Note that nothing comes free: this will increase the amount of time you spend at the nest.

If a nest is found after egg-laying is completed, you may need to estimate egg-laying date by floating the eggs in lukewarm water. Use a small transparent jar for this purpose (honey and jam jars work best). Hold the egg firmly between your fingers whilst placing it in the jar: do NOT drop the egg (***Figure 2***). The jar should be wide enough to let you

hold the egg firmly, and short enough to allow safe removal of egg with your fingers. Do NOT roll (or pour) the egg from the jar. It is a good idea to wipe the egg to remove water droplets, and float only one or two eggs of a clutch.

***Figure 2**. Placing an egg into a floating jar.*

We use two methods for estimating the number of incubated days (see also Fraga & Amat 1996).

(i) Noszály & Székely (1993)

*Table 1*. (a) Variation in floating position of the Kentish Plover egg during incubation. (b) Floatation stage off eggs in relation to the number of days incuabted. Eggs were measured daily from the date of laying. (Miklapuszta records, 1991–1992)

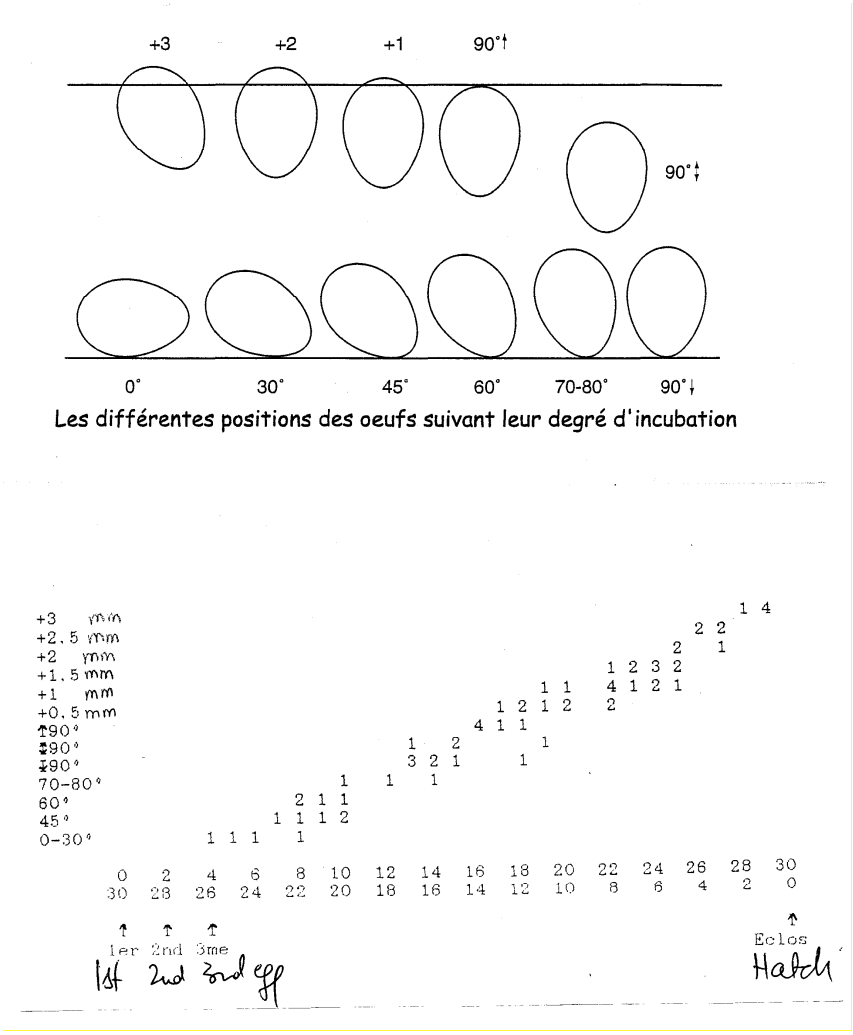
| | A tojás pozíciója és hossztengelyének a vízszintessel bezárt szöge<br>position of egg and angle of its longitudinal axis horizontal | lebegési stádium<br>stage of floating |
|---|---|---|
| | az edény alján fekszik<br>lays on the bottom of the dish<br>0 | A |
| | az edény alján fekszik, a hegyes vég leér, de a tompa vég nem emelkedik fel<br>the pointed edge approaches the bottom, but the blunt edge has not risen<br>< 45 | AB |
| | az edény alján fekszik, a hegyes vég leér, a tompa vég felemelkedik<br>the pointed edge is on the bottom, the blunt edge takes off<br>< 45 | B |
| | az edény alján fekszik, a hegyes vég leér, a tompa vég emelt<br>the pointed edge is on the bottom, the blunt edge erected<br>45 < és < 90 | C |
| | áll az edény alján, a hegyes vég leér<br>the egg stands at the bottom on the pointed edge<br>90 | D |
| | lebeg<br>the egg takes off and floats | E |
| | a tompa vég a felszínre bukkan, a tojás úszik<br>the blunt egg appears on the water surface | F |

These flotation stages correspond to the number of incubated days as calibrated in Southern Turkey (J Kis, unpublished data). Note that stage F can be anything between 10 days and 25-27 days:

| **Incubation stage** | **A** | **AB** | **B** | **C** | **D** | **E** | **F** |
|---|---|---|---|---|---|---|---|
| **Number of days incubated** (mean ± SE) | 0 ± 0 | 0.8 ± 0.3 | 2.4 ± 0.4 | 5.0 ± 0.7 | 8.0 ± 0.7 | 10.0 ± 0.8 | 11.2 ± 1.2 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Number of eggs** used for calibration | 5 | 7 | 7 | 6 | 3 | 3 | 3 |

(ii) Olivier Pineau (Tour du Valat Biological Station, France) has designed the following chart:




It is a good idea to write down a concise description of nest location in your Nest notebook (see below), the distances from landmarks (for instance, a small bush, grass patch or a peculiar piece of rubbish), and make a sketch. This may seem old fashioned given the accuracy of handhold GPS devices, although experience shows that the notes help visualising the spatial distribution of nests so that make fieldwork efficient. For remembering digits of coordinates may require photographic memory, whereas associating a nest with a particular landmark is easy: *'S6 is 4 m south from the blue shoe'*. If you decide to use UTM coordinates these will give you the distances in meters.

A plastic straw at a sufficient distance from the nest (e.g. 10 - 15 m) in a standard direction will speed up relocating the nest. Be sensible and remove straws if you suspect that people (or clever predators) may use them as clues. Collect the straws once all chicks have hatched or the nest has been predated. Straws are not foolproof either: grazing sheep and cattle often fancy chewing them.

Nests may be checked at 4-5 days intervals to estimate egg survival. If possible, stay at a distance from the nest and don't handle the eggs. Near the time of hatching

(approximately after the 22nd day of incubation), it is a good idea to check nests daily and tap the eggs gently. To ring the downy chicks in the nest-scrape, you may need to check peeping eggs 2-3 times each day. Eggs may peep for 2-3 days before hatching, although some peculiar chicks may pop out without much peeping. Near hatching you may also notice minute cracks on the eggshell; these may be sensed by gently turning the eggs between your fingertips. Tiny eggshell remains in the bottom of nest scrape let you identify successful nests for a few days after the chicks left the scrape. In contrast, chewed pieces of eggshell or egg remnants around the nest are telltale of predation: to figure out the culprits you need to use nest cameras.

We devote a full (or half) page for each nest in our Nest notebook, so that all data for a given nest are on a single page (***Appendix 2***). Bear in mind that incubation often speeds up toward the breeding season, so that nests laid late in the season may hatch faster than you expect. Small clutches (1 or 2 eggs) also have the tendency to hatch sooner than clutches of three.

## Trapping at the nest

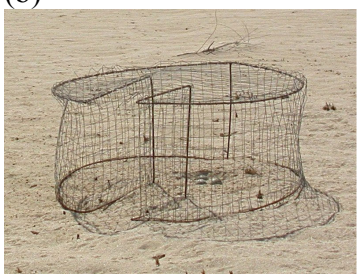
Equipment needed: traps, bird bag, binoculars and/or spotting scope.

Shorebird biologists use a variety of methods to trap plovers, including noose mats, mist-nets and funnel traps (see Conway & Smith 2000 for references). We found funnel traps by far the most reliable, safe and easy. Not all plovers can be caught; it is best to start with a simple method and get complicated ONLY if it is essential to trap a given individual. Bear in mind that the harder you push a parent the higher the chance it will abandon the nest.

(i) Funnel trap. The diameter of the trap is about 50 cm, and its height about 20-25cm (***Figure 3***). Use a local blacksmith to weld a frame from strong wire, and cover the frame with chicken wire of mesh size < 3cm in diameter. All sharp edges of the chicken wire should point outward to avoid injuring the trapped plover. It is good practise to check the traps weekly to make sure that all sharp edges remain outward. There is a slight preference for allowing the entrance width to vary (***Figure 3b & c*** *versus* ***Figure 2a***), to accommodate the needs of very cautious and crafty plovers that runs in-and-out the trap.

(a)

(b)



(c)

***Figure 3.*** *Funnel traps.*

1. Place the trap on the nest. You may position the nest in one of the 'corners' of the trap (***Figure 3c***), or in the middle (***Figures 3 a & b***). The former is safer in terms of trapping the parent, although it may take longer for the parent to enter the trap.
2. Hide at least 50m away from the nest to have a good view of the trap entrance. Watch out for humans, livestock herds and predators so they do not damage the trap, and/or the parents.
3. If a parent has entered the trap and sat on the eggs, you should run to remove it from the trap: quick actions will reduce stress and the chance of injury. When trying to grab the parent inside the trap you need to be careful to avoid damaging the eggs, or injuring the plover or yourself.

Females are usually easy to catch early in the morning and males just before dusk. Do NOT trap at extreme weather conditions such as raining, scorching heat or piercing cold. If it is essential to trap during the heat of the day, you either (i) shade the eggs by placing a flat object on the top of the trap (dry cow dung just works fine), or (ii) replace the eggs with dummies. Traps should not be left on the nest for excessive periods - the definition of 'excessive' is up to you, but it is rarely sensible to go beyond 20-30 minutes. Instead of forcing your way through, it is better to repeat trapping 2-3 days later.

You should NOT trap at a nest that has been incubated for less than 4-5 days. Also, if both parents happen to enter the trap simultaneously, release one immediately to let him/her incubate the nest whilst you measure and ring the other.

(ii) Round trap. If the funnel trap fails, you can try the round trap (***Figure 4***) on a different day. It takes longer to set up than the funnel trap, and you will need more time to retrieve the fishing line. Also, bear in mind that you may need special permission to use this trap.

To make a trap you need a ring (approx 80 cm diameter) of wire (or iron) about 0.5 cm diameter. Attach a loose fishing net to the ring using a threat all around - the less visible the net the better. Avoid shiny materials. You will also need a stick (reed or bamboo, about 40 cm), 2-3 pegs to hold the circle firmly on the ground, and fishing line with a reel to hold about 100 m of fishing line.

Set up the trap about 15 cm from the nest so the ring is well above the nest. Attach the fishing line to the stick, and firmly hold the other end of the fishing line in your hide (or car). After the parent has resumed incubation, pull the fishing line with one quick and strong motion so that the ring falls to the ground. Make sure that the pegs are strong enough to keep the trap in its place, and the fishing net is loose enough that the parent will not be injured.

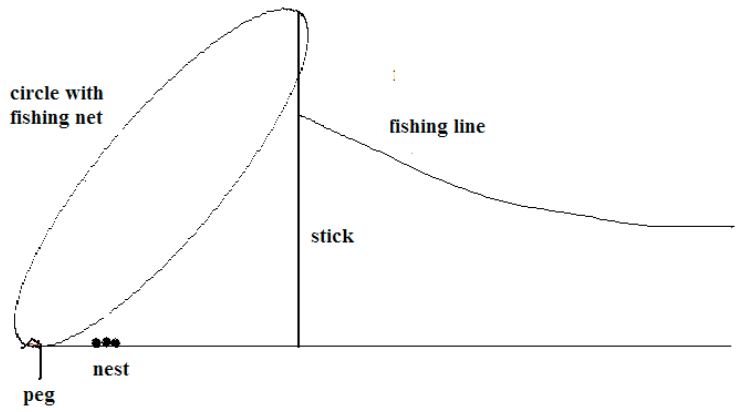



***Figure 4.*** *The round trap.*

## Ringing and measuring

Equipment needed: Metal rings, colour rings, ringing pliers, sliding callipers, wing ruler, spring balance, Capture notebook.

Ringing should follow the general protocols for a given country. Please stick to the rules. A useful reference is Redfern & Clark (2001). Before ringing plovers, especially freshly hatched chicks, you need to get advice from a trained ringer. As with nests, work efficiently during ringing; release the bird as soon as possible.

***Appendix 4*** summarises the main data we advise collecting from plovers. Kentish plovers usually live in saline environments, therefore rings made of non-corrosive material (e.g. steel) are preferred over aluminium ones. You may put the metal ring above the 'knee-joint' to reduce corrosion.

Darvic colour rings are resistant to sunlight and environment, and we prefer to use overlapping colour rings (as opposed to split rings): **W**hite, **Y**ellow, **O**range, **R**ed, **G**reen, light **g**reen and dark **B**lue are easily distinguishable. Avoid black, dark brown and light blue. Countries may have their own colour ringing scheme, although overall, we do not recommend using more than four rings on a given plover (***Figure 5,*** three colour rings and one metal ring). Leg flags are spreading among shorebird biologists: the *pros* and *cons* of leg flags need to be established. The safest ring position we found was one ring each above and below the 'knee-joint' on each leg. Chicks younger than three weeks should only be fitted with a metal ring and one colour ring at most. Note that colour rings may attract the attention of predators and bright colours such as Orange and Yellow are best avoided for chicks.

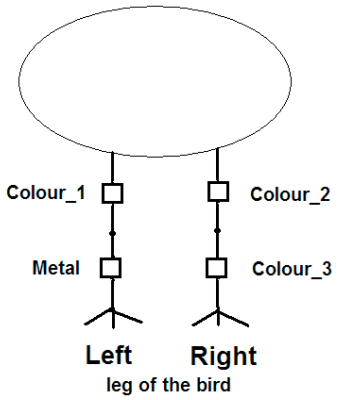



***Figure 5.*** *Schematic illustration of a coding system. The sketch shows a plover from behind.*

You may start a series with a given ring in Colour_1 position. Then choose Colour_2 and Colour_3 using the following chart, and cross the already used combinations:

| Colour_3 \ Colour_2 | B | G | g | O | R | W | Y |
|---|---|---|---|---|---|---|---|
| B | | | | | | | |
| G | | | | | | | |
| g | | | | | | | |
| O | | | | | | | |
| R | | | | | | | |
| W | | | | | X | | |
| Y | | | | | | | |

The scheme can be noted as *XX.XX*|*XX.XX* where *X* indicates a colour (or **M**etal) ring, the full stop marks the position of 'knee-joint' and vertical line divides the left and right leg. Thus the readout is *left above* . *left below* | *right above* . *right below*. For example, if Colour_1 is Blue, then the above plover is coded as B.M | R.W

This scheme may give 7 x 7 x 7 = 343 individuals. By rotating the location of Metal ring you can ring up to 1372 individuals.

**Appendix 5.** provides a guide how to take body measurements.

# Blood and tissue sampling

Equipment needed: sterile needle (size 25G or 23G), glass capillary, tubes with buffer or ethanol, small piece of tissue paper.

Blood sampling: Blood sampling is one of those things you need to see in person. We do our best to describe the methods here and provide pictures for the important steps, but this may not replace the demonstration by an experienced person. Before you think about practical issues, make sure that you have all necessary permissions: blood/tissue sampling permit, if you work in a foreign country hen you will need export and import permits. Bear in mind that it can take several months for the authorities to issue these permits, so submit the applications well in advance of the field season. With the

emergence of avian influenza, there will be regulations that need to be heeded, on top of the usual legislative, health and safety issues.

From adults take the samples from their brachial (wing) vein. First, open the left wing of the plover whilst holding it on its back (if you're left-handed, this may be the right wing). Then push apart the inside wing covers to make the area clearly visible around the wing vein. Gently wetting the inside wing covers helps to clear the area around the vein. This vein can be spotted as it crosses over a wing-bone. Second, hold a sterile needle flat on the wing, and with a single move puncture the vein; do NOT insert the needle into the vein itself. The rule is to pierce ONLY if you clearly see the vein; do NOT pierce by trial & error: this could easily injure (or kill) the bird. Suck the drops of blood into a capillary - usually a couple of droplets come out. Press the tissue paper on the wound, hold for about 20 s and fold back the wing to the body with the tissue on the wound. Bleeding usually stops within a few seconds. However, check before releasing the bird that the bleeding has stopped.

***Figure 6.*** *Blood sampling of adult plovers. Upper row: stretching the wing (left) and locating the brachial vein (middle), puncturing the vein with a 25G needle (right). Lower row: Filling the capillary with 25 µl of blood (left), stopping the bleeding by pressing a tissue to the wound (middle,) and after 20 s folding the wing with the tissue on the wound to the body (right).*

From chicks take blood samples from their leg vein. If you look carefully at the leg of a chick, you will see the vein goes along the inside of the tarsus at the 'knee-joint'. Carefully puncture the vein and collect about 25µl (1-2 droplets, a third of a 75 µl capillary) of blood. Make sure that the needle only pierces the vein and does NOT penetrate the bone or the muscles. Press a small piece of tissue paper on the wound to stop bleeding. Do not wipe off the blood with the tissue as it removes already coagulated blood from the wound and bleeding starts again. Empty the content of the capillary into an Eppendorf tube that contains Queen's lysis buffer (QLB, Seutin et al. 1991), or a rubber sealed tube that contains pure ethanol. This is most easily done by either gently blowing the blood out of the capillary or more hygienically, using a pipette aid.

***Figure 7.*** *Blood sampling from chicks.*

There are several ways to store the blood for genetic analyses; we recommend using QLB or ethanol. The DNA is preserved in both cases for many years, and it can be extracted using automated methods. Ethanol is cheap and can be obtained in most pharmacies. However, ethanol is inflammable and airline carriers might refuse to transport your samples stored in ethanol. Ethanol also evaporates easily, which means you have to make sure that the tube is tightly closed with the rubber sealed cap. QLB needs to be prepared in a lab before the field season (protocol after Yezerinac see ***Appendix 9***), and aliquoted into 1.5 ml Eppendorf tubes. It's not inflammable, does not need to be refrigerated in the field and is easier to handle in the lab and therefore our preferred buffer. If you use QLB make sure that you do not exceed the ratio of 25 µl of blood per 1ml of buffer. Otherwise the DNA will not be protected and may start degrading over time.

The sample tubes should be carefully labelled: ring number, date, location and sex/age (if known). Samples can be stored at room temperatures for several months, although a refrigerator is preferred. Ethanol samples are best stored at –20 ºC – +4 ºC for long term. Don't store them below these temperatures, because the ethanol will freeze and the DNA might be damaged.

Tissue sampling: Tissue sampling is particularly useful for dead plovers or embryos. We usually take a tiny piece of leg muscle and preserve it in ethanol. A few $mm^3$ tissues are enough. DNA in dead bodies degrades fast particularly in warm environments due to the work of enzymes such as DNAses. The best is to make sure that (some) DNA is preserved is by cutting the tissue with a sterile blade into small pieces so that the cells get smashed, and get in contact with ethanol which inhibits the enzyme activity. The storage conditions are the same as for blood.

## Trapping with chicks

Equipment needed: traps, tea-sieve, bird bag, binoculars and/or spotting scope.

It is possible to trap parents with chicks up to about 2 weeks of age. First, you need to catch ALL chicks in a brood, and carefully place the chicks under a tea-sieve (or strainer) large enough for all chicks. The sieve needs to be fixed on the ground with 2-3 pegs. Second, place a trap (funnel or round) over the sieve as you would do with the nest (***Figure 3c***). You may cover the bottom 10-15 cm around the trap with mud (or plant leaves) from the outside to block the direct view of parents of their chick; leave the funnel entrance open, however. This tends to entice parents to enter the trap.

If you're lucky enough to trap both parents, then you should keep the chicks in a warm place to avoid overcooling, and release them at the same time as the parents. If one

parent was caught and the other parent is around the family, you may release the chick(s) whilst measuring the caught parent, because the other parent will take over brood care. You should release ALL chicks at the same time in the location where you caught them.

## Checking broods

Equipment needed: binoculars, spotting scope, notebook, GPS device, mobile hide or car

Chicks are precocial so that they often wander over kilometres from the nest. Therefore, it is challenging to establish whether the chicks have fledged or died. We recommend revisiting marked families every 2-4 days, and recording the number and sex of attending parents, and the number of chicks (***Appendix 6***).

If you capture a brood that has not been marked before, you can estimate the age of these chicks by using a formula (Székely & Cuthill 1999). Since tarsus grows approximately linearly until the age of 25 days (as opposed to body mass that initially drops, and then accelerates), linear estimates appear to be acceptable: AGE (in days) = 2.520 * TARSUS (in mm) - 48.341.

It is a good idea to spend at least 15 minutes with each family to count all chicks and to establish whether both parents are still attending the brood. Brood desertion may not be permanent, so you need a few visits to make certain of desertion by one (or the other) parent. Marking chicks with different colour rings can help to establish which chicks are still alive. Brood mixing does occur in the KP, thus it makes hard to separate mortality from adoption.

Broods can be efficiently checked and recaptured at night using a powerful spotlight. We also caught parents using the tea-sieve method at night. Most chicks, even those beyond 20 days, are brooded at night so that they are easy to count. In addition, capturing chicks at night appears less distressing than during the day. An extra bonus of captures at night is that next day the parents are not bothered about you, whereas chasing chicks during daytime may force the parents to move with their chicks to a different brood-rearing site. You need to work carefully at night: you must know your study site and the whereabouts of the plovers to avoid getting stuck, or squashing nests and families. Night time fieldwork has implications on safety: your best interest is to check this out.

## Resightings

Equipment needed: binoculars, spotting scope, GPS device, notebook, mobile hide or car.

To build up a dataset on the movements of individuals, it is a good practice to note the location of colour-ringed plovers (***Appendix 7***). Every time you spot a colour-ringed individual, we recommend noting basic information as well, for instance on behaviour and possible pair-bonds.

A very useful summary file is ‘BirdRef’ (***Appendix 8***) that includes the identity (e.g. ring number) of all family members. In addition to captures and recaptures, you may also use unambiguous resightings to establish the identity of a family member. BirdRef can be linked to different files (e.g. nests, captures, behaviour), so this file is often the

backbone of statistical analyses.

## Notebooks

It is good practice to have two A5-sized notebooks for fieldwork (one for nest data i.e. Nest notebook, and one for captures i.e. Capture notebook), and a one-page-a-day diary. In the latter you may also note brood observations and resightings. In the diary, it is a good idea to record the major activities relevant to a given day (e. g. **5 May**: 5.50 start fieldwork; 5.55 trapping at B1/2 nest, 6.15 measuring & ringing female at B1/2....). These notes can be essential when you need to clarify the circumstances of an experiment, or reconstruct the details of an important observation.

********** **Disclaimer** **********

Please note we will not take responsibility for any consequences of the use (or misuse) of this guide. You need to check the regulations and legislations in your country and where fieldwork is carried out. We did not deal with many essential conceptual and practical issues for successful fieldwork (e. g. research hypotheses, experimental design, experimental protocols, logistics): to overcome these hurdles you need to be innovative. Good luck!

**********

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***Appendix 1.*** The mobile hide.

# Using a mobile hide in wader research

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Székely, T., Kis, J. & Kosztolányi, A. 2003. Using a mobile hide in wader research. *Wader Study Group Bull.* 103: 40–41.

We describe a mobile hide (or blind) that we designed for making observations of breeding plovers. We recommend it as being particularly suitable and flexible for studying waders in open habitats.

## INTRODUCTION

Researchers often investigate the behaviour and ecology of waders using hides (or blinds). Ideally, these should be mobile since waders (or their nests) may be scattered, and observations may be collected in several locations. For instance, the parents may leave their territory to feed, or the precocial chicks may move to distant areas. For these reasons, research workers often use motor vehicles such as cars, particularly all-terrain vehicles. However, these can be expensive; moreover their chassis can easily overheat and the noise of vehicles may disturb the birds. An additional source of disturbance may arise if researchers get out the vehicle, for instance to catch waders or to check their nests. To overcome the limitations of motorised vehicles, we developed an inexpensive and convenient mobile hide for our studies of Kentish Plovers *Charadrius alexandrinus* in Southern Turkey. For details on the study site and methods see Székely & Cuthill (2000), Kosztolányi *et al.* (2003) and Lendvai *et al.* (2004).

We focus on using the mobile hide in breeding ecology and behaviour, although it might be worth considering applications in studying migratory shorebirds at stopover sites or coastal wintering areas. This hide is probably best suited to studies of waders in open terrain where the substrate is fairly hard.

## THE MOBILE HIDE

The mobile hide had three main components: the frame and accessories, the wheels and the cover. First, the frame was made of 20 × 20 mm square-profiled iron (Fig. 1a). The frame ended in two forks at the front (see Fig. 1.). The lateral branches of the forks were made of 500 × 20 × 5 mm iron (L × W × H). Two small horizontal plates at the rear stopped the frame from sinking in the mud. The observer sat on a wood bench of 1050 × 250 × 30 mm (L × W × H) that was put across the bottom bars. We also fixed a basket of 300 × 170 × 250 mm (L × W × H) on the frontal and lateral middle bars, and 2–4 hooks on the top bars to hold bags, traps, tripods and binoculars. The frame, the basket and the hooks were all painted with rustproof metal paint.

Second, one bicycle wheel of 635 × 38 mm diameter with standard road tires was screwed to each fork so that the frame stood in an upright position (Fig. 1.). The observer moved the hide by gripping the bottom bars (either lateral or rear), and pushing the hide forward or backward. Before the hide was moved, the bench was pushed forward to let the observer walk.

Third, a hessian (or burlap) cover was made to cover the frame, and attached to it with straps (Fig. 1b). On each side of the hessian cover, there were two window slits; the top slits were used when the observer stood or walked, and the bottom ones were used when the observer sat down. When the slits were not in use, they were covered by roll-down hessian flaps from the inside and strapped to the cover. Three corners of the cover were not fully sewn down to the bottom: one slit of 1700 mm in the rear was left open for the observer to enter the hide, and two slits of 900 mm were left free for the wheels. The total weight of the hide was about 20 kg.

## USING THE MOBILE HIDE

Mobile hides constructed according to this design were indispensable to our fieldwork. They were relatively inexpensive (total cost: about 200 €), and made locally using simple materials. They were very effective, and caused less disturbance (and probably less stress) than motor vehicles. Four hides were spread over the study site so that they were stationed about one km apart. We only used a car to reach the hides, and to relocate them to different parts of the study site as necessary. We used the hides for the following tasks.

First, we searched for Kentish Plover nests and checked nests from the hide (Fig 2). When an incubating plover was spotted we carefully approached its nest, and measured the eggs whilst we stayed inside the hide. Care was taken to avoid trampling the eggs. Plovers were tolerant of the approaching hide; for instance a female only left her nest when the hide was less than one metre from her. After the hide was pushed away from the vicinity of nests, the parents quickly resumed incubation.

Second, we trapped parents at their nest using funnel traps. The trap was put above the nest, and after the parent went inside the trap, the hide was slowly pushed over the trap and the parent was gently removed from it. Captured plovers were measured and ringed in the hide away from the nest. This procedure was less disruptive than standard nest-trapping when the researcher walks (or runs) to remove the trapped wader.

**Fig. 1a.** The frame of the mobile hide (black lines) and the accessories (grey lines). Measurements are in mm.

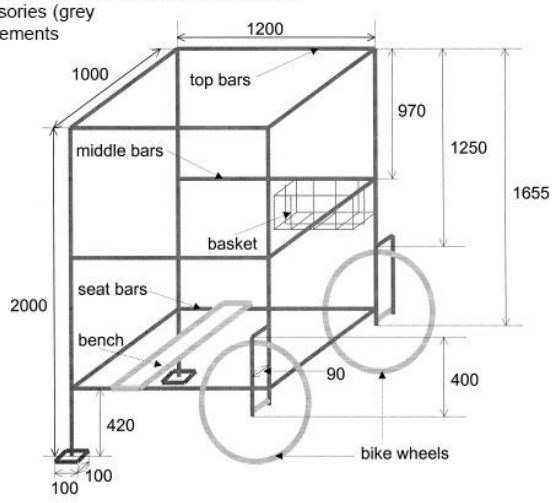


**Fig. 1b.** The hessian cover of the mobile hide (grey lines). One of the roll-down flaps is indicated by dots. The thick dotted lines represent middle bars of the iron frame.

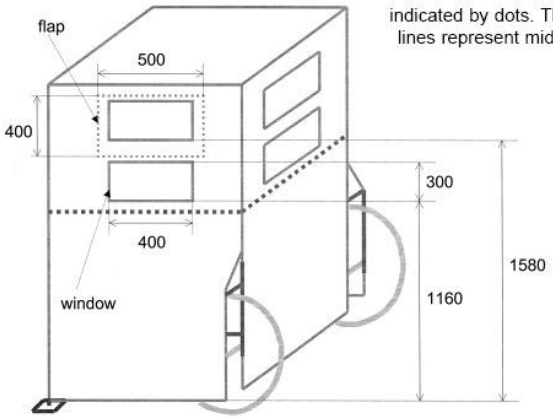


**Fig. 2.** The mobile hide in (**a**) standing position, and (**b**) in mobile position. Photos by T. Székely.



Third, we carried out various behavioural observations from the hides (Székely & Cuthill 2000, Kosztolányi *et al.* 2003). Even secretive behaviours, such as brood attendance and courtships, were easy to record, and the plovers appeared to behave naturally. A telescope was mounted on a middle bar using a window-mount (Fig. 2b), and it remained there while the hide was being moved. The mobile hide was particularly handy when the plover(s) under observation waded across mudflats, shallow shores, ditches or thick vegetation, since the observer was able to follow the bird monitoring its behaviour without disruption using the hide. Note that particularly strong winds may make the hide unstable and difficult to manage and manoeuvre.

All things considered, we strongly recommend this mobile hide for wader researchers, since it is more economic and effective than motor vehicles, and causes less damage to the habitat. Unlike hides or blinds that are commercially available and designed for hunters, fisherman or photographers, our hides were easy to move around. Moreover the hessian cover both shades the observer from direct sunlight, and also allows a breeze to blow through the hide and reduce discomfort in hot weather. If the hide is used in locations where the weather is cold and/or wet, we recommend using a waterproof canvas cover instead of hessian.

## ACKNOWLEDGEMENTS

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***Appendix 2.*** Nest records. First, you need to note all data for a given nest on a separate page (or half-page) in your Nest notebook. These will include nest location, egg measurements, dates of nest checks and nest fate. Second, you may type these data in a spreadsheet that looks like this:

| Year | Site | Nest ID | Latitude | Longitude | Found date | Laying date | End date | Fate | No. chicks | Clutch size | L1 | B1 | S1 | L2 | B2 | S2 | L3 | B3 | S3 | Observer | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1996 | A | 1 | 59625 | 89652 | 420 | | | HAT | 3 | 3 | 31.7 | 22.7 | E | 31.9 | 22.3 | D | 32.0 | 22.1 | D | AK | |
| 1996 | A | 2 | 59827 | 89636 | 420 | | 425 | PRED | 0 | 2 | | | AB | | | AB | | | | TS | |
| 1996 | A | 3 | 59324 | 89638 | 420 | | 425 | PRED | 0 | 2 | | | | | | | | | | JK | |
| 1996 | A | 4 | 58438 | 89024 | 429 | 427 | | | | 3 | | | AB | | | AB | | | AB | TS | |
| 1996 | A | 5 | 58603 | 88964 | 420 | | 515 | PRED | 0 | 3 | | | D | | | D | | | E | TS | |

YEAR
SITE – it is a good practice to divide the study site in small units that are refereed as 'sites'
NEST ID – give consecutive numbers to each nest for a given site
LATITUDE – GPS coordinate; use UTM coordinate system, if possible
LONGITUDE – GPS coordinate
FOUND DATE – the date the nest was found
LAYING DATE – the date of the laying of the last egg (either known or estimated using floatation stages)
ENDDATE – last date when the nest was checked
FATE – hatched/failed for one reason or the other
NO. CHICKS – number of chicks that hatched
CLUTCH SIZE - maximum number of eggs
EGG LENGTH (L1, L2 & L3) – length of each egg as measured using sliding calliper
EGG BREATH (B1, B2 & B3) – breath of each egg as measured using sliding calliper
EGG FLOATATION (S1, S2 & S3) – floatation stage of each egg, if laying date is unknown (separately for each egg)
OBSERVER
COMMENTS

***Appendix 3.*** Nest summary**.** It is often helpful to make a summary table on the last few pages of Nest notebook. This allows you to get updated how many nests you found, when the expected hatching dates are, and how many of nests failed or hatched.

Site: Salt marsh

| Nest ID | Laying date | 22nd day of incubation | Hatching date | Fate | Comments |
|---|---|---|---|---|---|
| 1 | 411 | 501 | 507 | Hatched | |
| 2 | 412 | 502 | | Predated | |
| 3 | 419 | 509 | | Predated | |
| 4 | 410 | | | ? | |
| | | | | | |



Site: Sand dune

| Nest ID | Laying date | 22nd day of incubation | Hatching date | Fate | Comments |
|---|---|---|---|---|---|
| 101 | 420 | 510 | | Hatched | |
| 102 | 416 | 510 | 513 | Hatched | |
| 103 | 414 | 504 | | ? | |
| 104 | 415 | 505 | | Predated | |
| | | | | | |

***Appendix 4.*** Capture notebook. You may use this table for both your field notes and your spreadsheet file

| Ring | Year | Site | Nest ID | Sex | Date | Time | Weight | Wing length | Tarsus length | Code | Observer | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DH83328 | 1996 | B | 12 | F | 418 | 550 | 43.0 | 118 | 29.8 | XX.MX \| XX.WX | JK | |
| DH83329 | 1997 | C | 31 | F | 418 | 635 | 41.6 | 111 | 28.7 | XX.MX \| XX.BX | AK | |
| DH83329 | 1996 | B | 83 | F | 510 | 1754 | 41.8 | 108 | 28.5 | XX.MX \| XW.OX | JK | |
| DH83329 | 1999 | D | -1 | F | 605 | 1834 | 45.8 | 112 | 28.5 | XX.MX \| XW.WX | AK | |
| DH83330 | 1996 | B | 14 | M | 418 | 620 | 40.6 | 115 | 28.6 | XX.MX \| XW.YX | AK | |
| DH83331 | 1996 | B | 31 | M | 419 | 2030 | 42.0 | 115 | 28.6 | XX.MX \| XG.BX | TS | |



RING – metal ring number
YEAR
SITE
NEST ID – same as in Nest file (you may use negative numbers for broods that were found after hatching)
SEX – M, F, J, for adult male, adult female and chick, respectively
DATE – date of capture
TIME – time of capture
WEIGHT – body mass (g)
WING LENGTH – measured by stretching the right wing (only adults and chicks older than 3 weeks, mm)
TARSUS LENGTH – the length of right tarsus (mm)
CODE – colour ring code in the form XX.XX | XX.XX
OBSERVER - ringer
COMMENTS – optional extra information about the bird

Make sure you do not duplicate the same information in different files. For instance, if nest coordinates are registered in the Nest file, there is no point including nest coordinates in the Capture file.

***Appendix 5***. Measurements of adults and chicks

In case of wings and tarsus we usually take the measurements of both right and left limb. This can give you information about the asymmetry of the birds, but serves also as a control to check your measurements.

**Tarsus**

Equipment needed: Vernier or dial calliper, notebook

We use the following technique according to Redfern and Clark (2001).

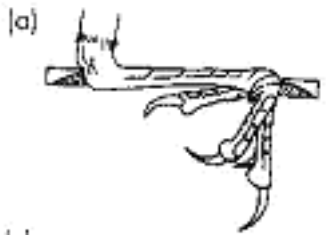


***Figure A5.1.*** The minimum tarsus method. Image from Redfern and Clarke (2001).

There is always some variation in tarsus measurements between people. To keep this variation at a minimum it is important to keep bird and leg in a standardised way and know exactly what you want to measure.
We measure the *minimum tarsus* of plovers. If you do it for the first time, take a moment of time to feel for the tarsus bone with your finger tip, before applying the calliper. Then hold the bird firmly in your hand, with the head between index and middle finger in the so called *ringer's grip*. The leg you want to measure should be aligned parallel to the main axis of the body. Tarsus and tibia should be in an angle of 90° as shown on the picture. The leg and three sides of the calliper (ruler and the two brackets) should form a rectangle. Fix the tarsus using your thumb and your ring finger. Open the calliper and press the bracket until the tarsus is firmly between the adjustable and firm bracket. Check the position of the bone, if it still as shown in ***Figure A5.1*** you are fine, if not readjust the leg. Then press the adjustable bracket gently until you feel some resistance by the end of the bone. Note the measurement to the nearest 1/10 mm. Repeat the process twice more and take the mean of your measurements.
Measuring the tarsus needs some practice. The point of resistance is usually the source of variation between measurements. This might lead to differences of up to 1 mm between people. At a total length of 25-30 mm of adults this is quite a lot. Remember that the tarsus length will not change in full grown adults, so the variation is entirely due to measurement variance. If you recapture adult birds later in the season, compare your

measurements with the previous ones. An accuracy of 0.2-0.3 mm is reasonable. For chicks measurement errors can be an even bigger problem, particularly if you want to measure growth rates.
As usual be considerate with the bird, you don't want to break it's leg. Also don't stress the plover to long by practising exhaustively to get the exact measurement. After some practise each measurement should not take more than two minutes.

**Wing**

Equipment needed: Wing ruler, notebook

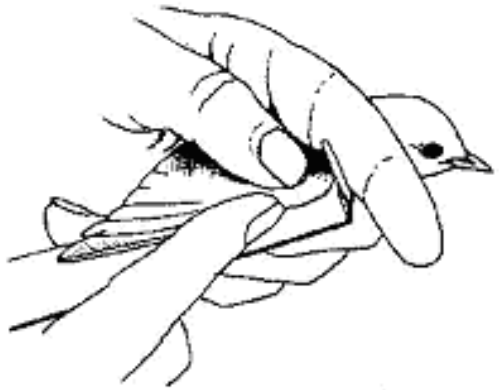

***Figure A5.2.*** Wing measurement using the maximum chord method. Image taken from Redfern and Clarke (2001).

We use the *maximum chord* method as shown in Redfern and Clark (2001). As with tarsus the position of the wing and bird are important. Hold the bird as shown in ***Figure A5.2***. Open the wing, straighten the primary feathers and carefully slide in the ruler as shown. Fold the wing back that it is as close to its natural resting position as possible. Again, the position of the wing in regards to the body is essential. Using your right index finger straighten the alula. Make sure that the alula falls into a line that the curvature of the wing is reduced (***Figure A5.2***). Take the measurement of the second primary,

that is the longest feather if not worn or replaced during moult. Make sure that the primaries are not broken or heavily worn. If they are make a note. Again, if you measure the wing length for the first time repeat the procedure to get an idea about your measurement error.

**Weight**

Equipment needed: Pesola spring balance, cone (different size for chicks and adults), notebook

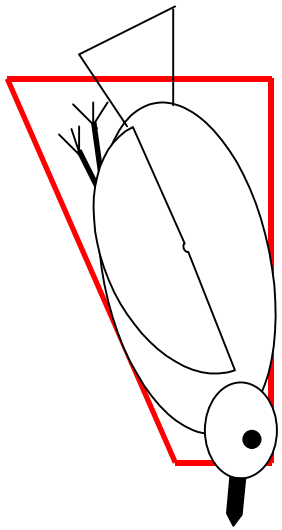

***Figure A5.3.*** Cone (in red) for measuring weight using spring pesolas.

Weight measurement is important because it can tell a great deal about the conditions of the birds. Females can sometimes carry eggs which you can feel by gently pressing their belly. (If you trap on the nest they shouldn't because you should not catch them before clutch completion!)
We use pesola spring balances to measure plovers, but it also possible to use electronic balances. Adult Kentish plovers have a body weight of 35 – 45 g, so a 50 g pesola spring is appropriate. A useful device to hold the bird during measuring with a pesola is a plastic cone that can be hung to the clip of the balance. Calibrate the spring before measuring the first plovers. When you are read gently force the bird into the cone with the feet stretched out towards the tail and the head pointing towards the narrow end of the cone (as shown in ***Figure A5.3***). If you position the bird as shown, it won't be able to struggle. Never hold the balance by the tube, but always at the hook or loop and make sure that cone and bird hang freely. For chicks you will

need a smaller cone. Note the weight. Regularly clean the cones after a couple of days measuring, since the birds often defaecate in this situation.

**Fat**

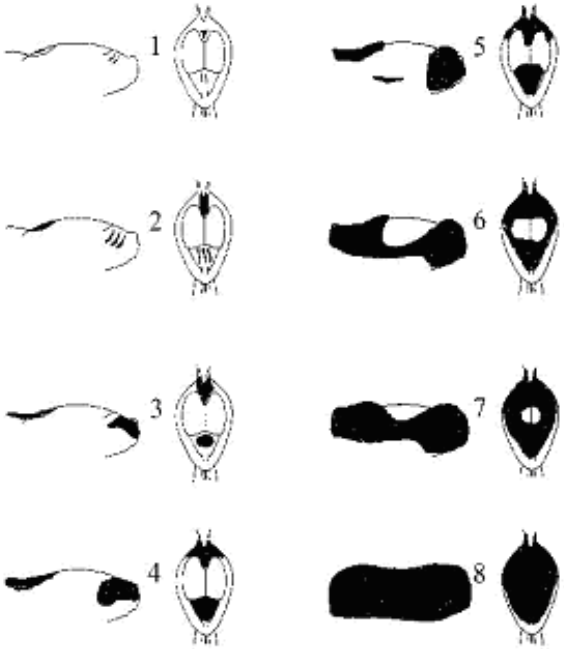


***Figure A5.4.*** Fat scores. The fat distribution is shown in black. Image taken from Redfern and Clarke (2001)

Fat provides an indicator about the condition of birds. To measure it, hold the bird with its feeting pointing towards you. With your free hand slightly extend its neck, that the furcula is visible. The furcula is located at the beginning at throat, indicated by the black triangle in ***Figure A5.4.1.*** Blow the feathers at the furcula aside. You need good light conditions to see the fat, especially if there is no or very little fat. Note the deposit according to the figure. Breeding birds usually have fat scores between zero and four.

**Bill**

Equipment needed: Calliper, notebook

This is an optional measurement that you may want to take. There are several measures that can be taken including bill depth and length. We usually measure bill length, which can in some waders identify sex or race. We measure it from the tip to the start of the feathers using a calliper to the nearest 1/10 mm.

***Appendix 6.*** Variables recorded for brood encounters.

| Year | Site | Brood ID | Date | Time | Parent | Chicks | Latitude | Longitude | Habitat | Observer | Notes |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1999 | B3 | -1 | 527 | 1054 | 4 | 3 | 58177 | 89026 | SALICORNIA | TS | |
| 1999 | B3 | -1 | 529 | 1123 | 4 | 3 | 58107 | 88682 | LAKE | JK | |
| 1999 | B3 | -1 | 531 | 545 | 4 | 3 | 57738 | 88998 | LAKE | AK | |
| 1999 | B3 | -1 | 602 | 2043 | 4 | 3 | 58156 | 89022 | LAKE | AK | |
| 1999 | B3 | 21 | 606 | 845 | 4 | 3 | 58022 | 89160 | LAKE | AK | |
| 1999 | B3 | 21 | 608 | 1858 | 2 | 2 | 58076 | 89034 | SALICORNIA | TS | |
| 1999 | B3 | 21 | 609 | 1633 | 4 | 3 | 57858 | 89145 | LAKE | AK | |



YEAR
SITE – see nest file
BROOD ID – brood identifier; negative signs indicate that the brood hatched from a nest we did not find
DATE – date of re-sighting
TIME – time of re-sighting
PARENT – number and sex of parents (4 – both parents, 3 – only male, 2 – only female)
CHICKS – number of chicks
LATITUDE – GPS coordinates; UTM coordinates are often more useful than other types
LONGITUDE – GPS coordinates; UTM
HABITAT - sensible description of habitat
OBSERVER
COMMENTS – notes & additional details

*Appendix 7.* Resightings of colour-marked plovers.

| Year | Site | Latitude | Longitude | Date | Time | Code | Sex | Observer | Comment |
|---|---|---|---|---|---|---|---|---|---|
| 1997 | C2 | 58637 | 88962 | 430 | 830 | Mg.XX\|OW.XX | M | AK | |
| 1997 | D | 58437 | 89104 | 430 | 910 | MO.XX\|RO.XX | F | TS | |
| 1997 | D | 58437 | 89104 | 430 | 910 | MW.XX\|RG.XX | M | AK | Courts XX.MG\|XX.RW |
| 1997 | D | 58419 | 89081 | 430 | 1020 | MY.XX\|gO.XX | F | AK | Former mate of Mg.XX\|OW.XX |
| 1997 | A | 58397 | 89087 | 430 | 1400 | MY.XX\|RY.XX | M | TS | |
| 1997 | C2 | 58386 | 89065 | 430 | 1425 | XX.MG\|XX.Rg | M | JK | |



YEAR
SITE
LATITUDE – GPS coordinates
LONGITUDE – GPS coordinates
DATE – date of re-sighting
TIME – time of re-sighting
CODE – colour ring combination
SEX – sex of observed plover
OBSERVER
COMMENTS – notes about behaviour

***Appendix 8.*** BirdRef: a useful summary of family members. Note that only those families are included that have at least one marked member. Negative nest numbers refer to broods that hatched from unknown nests.

| Year | Site | Nest | Lay date | Hatching date | Male id | Female ID | Chick1 ID | Chick2 ID | Chick3 ID |
|---|---|---|---|---|---|---|---|---|---|
| 1996 | A | -3 | | | | | 83673 | 83674 | |
| 1996 | A | -2 | | | | | 83670 | 83671 | 83672 |
| 1996 | A | -1 | | | | 83342 | 83343 | 83344 | 83345 |
| 1996 | A | 1 | | 504 | 83614 | 83616 | 83611 | 83612 | 83615 |
| 1996 | A | 6 | | 510 | 83665 | 83666 | 83667 | 83668 | 83669 |
| 1996 | A | 12 | | 519 | 83765 | 83761 | 83762 | 83763 | 83764 |
| 1996 | B | 12 | | | | 83328 | | | |
| 1996 | B | 31 | 410 | | 83331 | 83329 | | | |
| 1996 | B | 39 | 417 | | 83561 | 83560 | | | |
| 1996 | C | -4 | | | | | 85558 | | |
| 1996 | C | -3 | | | | | 83899 | | |
| 1996 | C | -2 | | | 83684 | | 83678 | 83685 | 83686 |
| 1996 | C | -1 | | | | | 83340 | 83341 | |
| 1996 | C | 4 | 408 | | 83554 | 83553 | | | |
| 1996 | C | 23 | 415 | | 83555 | 83822 | | | |
| 1996 | C | 25 | 413 | | 83559 | 83558 | | | |
| 1996 | C | 42 | 424 | | 83569 | 83570 | | | |
| 1996 | C | 43 | 423 | | 83339 | 83565 | | | |
| 1996 | C | 44 | 424 | | 83566 | 83567 | | | |

***Appendix 9.*** Protocol to prepare Queens Lysis Buffer (after Dr Stephen Yezerinac, Queens University Kingston, Canada)

Recipe for 1X strength

0.01 M Tris-Cl
0.01 M NaCl
0.01 M EDTA
1% (w/v) n-lauroylsarcosine (or n-lauroylsarcosine sodium salt which dissolves better)

adjust to pH=7.5 using NaOH

DO NOT AUTOCLAVE AFTER ADDITION OF n-lauroylsarcosine, WHICH BUBBLES-UP.

For example, to make 500 ml, enough for 500 blood samples: add to 400 ml
sterile distilled water, 0.61 grams Tris-Cl, 0.3 grams NaCl, 1.7 grams
EDTA, 5 grams n-lauroylsarcosine; stir until dissolved, then adjust pH to
7.5 using Sodiumhydroxide and bring volume up to 500 ml by adding sterile distilled water.
Dispense into 1 ml aliquots and use.